

Cloning and Characterization
of the
t-Cinnamic Acid Decarboxylase Gene
of *S. cerevisiae*

Vom Fachbereich Biologie
der Universität Oldenburg
zur Erlangung des Grades eines
Doktors der Naturwissenschaften (Dr. rer. nat.)
angenommene Dissertation

von
Peter Wolf Doerner
geb. am 28.12.53 in Manila

Oldenburg 1987

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1. Introduction

The decarboxylation of phenylacrylic acids to the corresponding styrenes by the yeast *S. cerevisiae* is a well-known, albeit somewhat obnoxious enzyme reaction. It was recognized as responsible for the phenolic off-flavor sometimes observed in the production of beer. In contrast to most laboratory strains of yeast, typical brewing strains have little or no ability to carry out this reaction (Wackerbauer 1978, Goodey 1982, Thurston 1981). However, many microorganisms, including so called wild yeast strains that frequently contaminate fermentations, are capable of this decarboxylation reaction (Wackerbauer 1978, Steinke 1964, Lindsay 1975, Albagnac 1975).

The substrates for this enzyme reaction are present in the mash, wort and juice used in fermentations of cereal or fruit. Cereal grains are rich in hydroxycinnamic acids (Slonimski 1980, Wall 1961). When corn is cooked for grain alcohol fermentations, some 4-OH styrene and 4-OH,3-OCH₃ styrene are already formed by thermal degradation of *p*-coumaric and ferulic acids, respectively (Steinke 1964). However, between two- and eight-fold more were produced in the subsequent fermentation by yeast (Steinke 1964). When malted barley is extracted with hot water, these acids are released and can give rise to phenolic off-flavors in beer upon brewing (Wackerbauer 1978, Goodey 1982).

Wort and mash are, however, not the natural habitat of yeast. The natural habitat of yeast is plant material, in particular decaying fruit. Hydroxycinnamic acids such as *p*-coumaric acid and their carboxymethylated derivatives such as ferulic acid are ubiquitous in higher plants (Goodwin 1983). Why did *S. cerevisiae* and other microorganisms evolve a capability to decarboxylate phenylacrylic acids?

Naturally occurring hydroxycinnamic acids exhibit antimicrobial activity. Baranowski *et.al.* (1980) found that caffeate, *p*-coumarate and ferulate inhibited growth of a Champagne strain of *S. cerevisiae* in a complex medium. Stahmann *et.al.* (1976) have shown that the germination of coffee rust uredospores is inhibited by *t*-cinnamic acid derivatives. Finally, Gupta *et.al.* (1976) identified ethyl *p*-methoxycinnamate as the main antifungal agent from the East Indian plant *Curcuma zedoaria*.

The ability to eliminate these inhibitory compounds confers a selective advantage on an organism utilizing plant material as growth substrate. To my knowledge, decarboxylating activity on phenylacrylic acids yielding the corresponding styrenes has only been observed in organisms living on

matter rich in plant phenolics. The capability for this reaction has been found in *Aspergillus niger* and *Penicillium glaucum* (Herzog 1908), a large number of *Enterobacteriaceae* (Lindsay 1975), *Aerobacter* spp (Finkle 1962), *Pseudomonas cepacia* (Andreoni 1984), *Cladosporium phlei* (Harada 1976) and *Polyporus circinata* (Bayne 1976). However, phenylacrylic acids are not the sole antimicrobial agents present in higher plants.

Although plants are continuously confronted with microorganisms, only few interactions with bacteria or fungi result in disease. Most interactions are beneficial to both sides involved. To cope with potentially deleterious or fatal interactions with microorganisms, higher plants have evolved inducible disease resistance mechanisms (Legrand 1983, Kolattukudy 1983, Bell 1981, Ryan 1983, Darvil 1984). Although the different resistance mechanisms usually act in concert, one of the more prominent defense mechanisms is the production of phytoalexins by the cells of the afflicted area. Phytoalexins are a diverse group of substances. One of the main biosynthetic routes leading to their formation is the shikimate pathway. *t*-Cinnamic acid is a key intermediate in this pathway.

A successful infection cannot be established before the plant's resistance mechanisms are overwhelmed. A plant pathogen usually overcomes the inhibitory phytoalexin by metabolizing it (VanEtten 1983). Generally, the lipophilic phytoalexins are metabolized by oxidation to more polar compounds (VanEtten 1983), because these can be eliminated with greater ease. However, *Botrytis cinera* eg. metabolizes a substituted pterocarpan phytoalexin by methylation, resulting in a more volatile product (Ingham 1976).

A fundamental strategy to overwhelm phytoalexin resistance mechanisms would be a sequestering of early intermediates, thus rendering all following steps impossible. Metabolization of phenylacrylic acids to the corresponding styrenes would be a straightforward way to manage this. The phenylacrylic acid intermediates participate in plant metabolism in their biochemically activated form, as thioesters of coenzyme A. The presence of a carboxylic function in this class of molecules is therefore essential. Styrenes could not further participate in biosynthesis of L-phenylalanine derived phytoalexins because they cannot be activated biochemically to thioesters. Furthermore, they are quite volatile, so that their elimination is facile. It is probably no coincidence that some of the species possessing a phenylacrylic acid decarboxylase are plant pathogens or saprophytes.

Further to their role in phytoalexin biosynthesis, phenylacrylic acids pos-

sess more fundamental functions. They are key intermediates in plant secondary metabolism. Pathways leading to diverse natural products such as lignin, flavonoids, isoflavonoids, coumarins, lignans and some alkaloids originate from *t*-cinnamic acid, which is produced in plant cells from L-phenylalanine by L-phenylalanine ammonium lyase (PAL) (Goodwin 1983). Members of all of these product classes are synthesized under normal growth conditions. The elimination of the intermediate *t*-cinnamic acid, which can be considered as a biochemical sink, can easily be envisioned as wreaking havoc on a variety of cellular functions.

The arguments developed above show, that it can be of great importance for an organism living off plant matter to possess a phenylacrylic acid decarboxylase. Further to the ability to detoxify a class of growth inhibitory lipophilic acids, which would be crucial for a saprophyte, the ability to eliminate an intermediate involved in plant secondary metabolism as well as in defense mechanisms would constitute an immense advantage for a plant pathogen as well.

What is known about the diverse phenylacrylic acid decarboxylases? Most investigations have dealt with the yeast enzyme, although the enzymes from *Cladosporium phlei* (Harada 1976) and *Aerobacter* sp (Finkle 1962) have been characterized in some detail. The enzyme has been purified severalfold only from *Cladosporium phlei* (Harada 1976) and from *Polyporus circinata* (Bayne 1976).

An evaluation of substrate specificity reveals, that the enzymes can be classified into two groups. The enzymes belonging to group I are unable to decarboxylate *t*-cinnamic acid, but hydroxylated arylacrylic acids, or hydroxylated *t*-cinnamic acid analogs which are substituted even further on the aryl moiety, will serve as substrates. The enzymes characterized from *Aerobacter* spp (Finkle 1962), *Cladosporium phlei* (Harada 1976) and from *Polyporus circinata* (Bayne 1976) belong to this group. The enzymes from these three species are expressed constitutively and possess pH optima around pH 6. The addition of *t*-cinnamic acid reduces the amount of CO₂ evolved from *p*-coumaric acid to half, when the assay is performed with the enzyme from *Cladosporium phlei* (Harada 1976). No data was available on substrate specificity in respect to *t*-cinnamic acid for *Pseudomonas cepacia* (Andreoni 1984).

Group II is characterized by the ability to utilize *t*-cinnamic acid as substrate. The enzymes from *Saccharomyces cerevisiae* (Albagnac 1975; Goo-

dey 1982; Megnet, unpublished results) and from *Fusarium* sp (Megnet, unpublished results) belong to this category. The optimal pH for decarboxylation for the yeast enzyme was found to be pH 6.5 (Goodey 1982). Albagnac (1975) found a broad spectrum of hydroxylated and carboxymethylated *t*-cinnamic acid derivatives that would serve as substrates.

With the exception of Tubb's group (see eg Goodey (1982)), very little work has been done on the genetics and molecular biology of *t*-cinnamic acid decarboxylase. These authors have worked with laboratory and brewing strains of *S. cerevisiae*. They have found that the gene for *t*-cinnamic acid decarboxylase is probably located in the nucleus of the yeast cell (Goodey 1982) and that the wild type gene is dominant.

In this work, the nature of the growth inhibitory action of *t*-cinnamic acid on yeast, as well as the genetics and molecular biology of the gene encoding *t*-cinnamic acid decarboxylase in *S. cerevisiae* were studied in greater detail.

2. Materials and methods

2.1. Media and buffers

2.1.1. Media

All amounts given are for 1 l medium. For plates 15 g agar were added. Antibiotics were added after autoclaving as follows: Ampicillin to 75 $\mu\text{g/ml}$ and tetracyclin to 10 $\mu\text{g/ml}$; stock solutions of antibiotics were prepared as described by Maniatis (1982). Amino acids and other supplements in yeast media were added as suggested by Sherman (1983); in *E. coli* media amino acids were added to 40 $\mu\text{g/ml}$, thiamine to 5 $\mu\text{g/ml}$ and thymine to 25 $\mu\text{g/ml}$ unless indicated otherwise. All other stock solutions were made up, pH adjusted when necessary, filtered through a .22 μm membrane and sterilized by autoclaving. Generally, Mg^{2+} , Ca^{2+} and PO_4^{2-} salts as well as carbon sources were added after autoclaving from sterile stock solutions.

LB (Maniatis 1982)

Tryptone	10.0 g
Yeast extract	5.0 g
NaCl	5.0 g

M 10 (Schleif 1981)

Na_2HPO_4	7.0 g
KH_2PO_4	3.0 g
NH_4Cl	1.0 g
0.1 M FeCl_3	.3 ml
after autoclaving add, when cool:	
1 M MgSO_4	1.0 ml
1 M CaCl_2	.1 ml
10 mM MnCl_2	5.0 ml
Carbon source	to 2.0 %

Minimal A (Miller 1972)

K_2HPO_4	10.5 g
KH_2PO_4	4.5 g
$(\text{NH}_4)_2\text{SO}_4$	1.0 g
Sodium citrate $\cdot 2\text{H}_2\text{O}$	.5 g

NB

Difco Nutrient Broth	8.0 g
NaCl	5.0 g

NBTT

Difco Nutrient Broth	8.0 g
NaCl	5.0 g
1 mg/ml thymine	10.0 ml
1 mg/ml trimethoprim made up in 5% acetone (boil, to bring into solution, then add immediately)	10.0 ml

SOB (Hanahan 1983)

Bacto tryptone	20.0 g
Bacto yeast extract	5.0 g
NaCl	.6 g
after autoclaving add:	
1 M MgSO_4 / 1 M MgCl_2	10.0 ml

SOC (Hanahan 1983)

same as SOB , with the addition of:	
2 M Glucose (sterile filtered)	10.0 ml

Yeast extract media

Yeast extract	20.0 g
after autoclaving add:	
1.5 M KH_2PO_4	5.0 ml
2 M MgSO_4	1.0 ml

then add for:

YEX

2 M Glucose	50.0 ml
-------------	---------

YG

87% glycerol	30.0 ml
--------------	---------

YGG

87% glycerol	30.0 ml
2 M Glucose	5.0 ml

Yeast extract / peptone media (Sherman 1983)

Yeast extract	10.0 g
Peptone	20.0 g
after autoclaving add:	
1.5 M KH_2PO_4	5.0 ml
2 M MgSO_4	1.0 ml

then add for:

YEPD

2 M Glucose	50.0 ml
-------------	---------

YEPG

87% Glycerol	30.0 ml
YEPDG	
2 M Glucose	5.0 ml
87% Glycerol	30.0 ml

YM (Croft 1973)Salts:

$\text{NH}_4\text{H}_2\text{PO}_4$	6.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	.5 g
$(\text{NH}_4)_2\text{SO}_4$	2.0 g
KH_2PO_4	1.0 g
NaCl	.1 g
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	.1 g
bidest H_2O	944.0 ml

Trace elements:

H_3BO_3	50.0 mg
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	40.0 mg
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	40.0 mg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	40.0 mg
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	20.0 mg
KJ	10.0 mg
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	4.0 mg
bidest H_2O	ad 100.0 ml

Vitamin solution:

Calcium pantothenate	40.0 mg
Thiamin · HCl	40.0 mg
Inositol	40.0 mg
Pyridoxine	40.0 mg
Nicotinic acid	10.0 mg
Biotin	.5 mg
bidest H_2O	ad 100.0 ml

autoclave the salt solution, then add from sterile stocks:

Trace element solution	1.0 ml
Vitamin solution	5.0 ml
2 M Glucose	50.0 ml

YMSsame as **YM** with the addition of:

Sorbitol	183.0 g
.15 g/ml Yeast Nitrogen Base	5.0 ml
2 M Glucose	only 20.0 ml

YMCS - xsame as **YM** with the addition of:

Sorbitol	183.0 g
.15 g/ml Yeast Nitrogen Base	5.0 ml
2 M Glucose	only 20.0 ml

Amino acids, adenine and uracil were added, when necessary, to the amounts suggested by Sherman (1983).

EMM 2 (Mitchison 1970 and Nurse 1975)Salt solution (x 25):

Potassium hydrogen phthalate	76.5 g
KCl	25.0 g
MgCl ₂	12.5 g
Na ₂ SO ₄	250.0 mg
CaCl ₂	250.0 mg

Carbon source:

Glucose	50.0 %
---------	--------

Nitrogen source:

NH ₄ Cl	100.0 g
--------------------	---------

Phosphate source:

Na ₂ HPO ₄ · 2H ₂ O	89.0 g
--	--------

Vitamines (x 100):

Inositol	1.0 g
Nicotinic acid	1.0 g
Calcium pantothenate	100.0 mg
Biotin	1.0 mg

Trace elements:

H ₃ BO ₃	50.0 mg
MnSO ₄ · H ₂ O	40.0 mg
ZnSO ₄ · 7H ₂ O	40.0 mg
FeCl ₃ · 6H ₂ O	20.0 mg
(NH ₄) ₆ Mo ₇ O ₂₄ · 4H ₂ O	110.0 mg
KJ	10.0 mg
CuSO ₄ · 5H ₂ O	4.0 mg
Citric acid · H ₂ O	109.0 mg

Autoclave a 1x salt solution then add per liter:

Nitrogen source	50.0 ml
Phosphate source	20.0 ml
Vitamins	10.0 ml
Trace elements	10.0 ml
2 M Glucose	for plates: 40.0 ml
	liquid media: 20.0 ml

EMM 2 S

same as EMM 2, but:

Sorbitol	218.6 g
----------	---------

EMM 2 CS

same as EMM 2, but:

Sorbitol	218.6 g
amino acids, adenine and uracil but without leucine; added to the amounts suggested by Sherman (1983)	

MEA (Booth 1971)

Malt extract	20.0 g
after autoclaving add:	
1.5 M KH_2PO_4	1.0 ml
2 M MgSO_4	.5 ml
2 M Glucose	5.0 ml

MEA + *t*-CA

same as MEA with the following addition:

100 mM <i>t</i> -cinnamic acid in absolute ethanol	6.0 ml
--	--------

Prespore medium (Sherman 1983)

Yeast extract	8.0 g
Bacto peptone	3.0 g
2 M Glucose	140.0 ml
2.4 mg/ml Uracil	10.0 ml
10 mg/ml Leucine	2.0 ml
10 mg/ml Histidine	2.0 ml

Sporulation medium (Sherman 1983)

Potassium acetate	10.0 g
Yeast extract	1.0 g
2 M Glucose	1.4 ml
2.4 mg/ml Uracil	10.0 ml
10 mg/ml Leucine	2.0 ml
10 mg/ml Histidine	2.0 ml

Fries (Booth 1971)

Ammonium tartrate	5.0 g
Ammonium nitrate	1.0 g
KH_2PO_4	1.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	.5 g
NaCl	100.0 mg
CaCl_2	100.0 mg
after autoclaving, add:	
Vitamin solution (YM)	5.0 ml
Trace element solution (YM)	1.0 ml
2 M Glucose	50.0 ml

2.1.2. Buffers**Z - Buffer: (Miller 1972)**

60 mM $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$	16.1 g
40 mM $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$	5.5 g
10 mM KCl	750.0 mg
1 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	246.0 mg
autoclave, then add the following just before use:	
50 mM β -mercaptoethanol	2.7 ml

ZY - Buffer:

Z-Buffer x2 w/o β -mercaptoethanol (Miller 1972)	500.0 ml
β -mercaptoethanol	2.7 ml
25% SDS	1.0 ml
40 mM Phenyl methyl sulfonyl fluoride (PMSF)	25.0 ml
2 M MgSO_4	500.0 μl
bidest H_2O	470.0 ml

20 x SSC:

NaCl	175.3 g
Na citrate	88.2 g
adjust pH to 7.0, autoclave to sterilize	

20 x SSPE:

NaCl	210.4 g
NaH_2PO_4	31.2 g
0.5 M EDTA	2.0 ml
adjust pH to 7.7, autoclave to sterilize	

100 x Denhardt's solution: (100 ml)

Ficoll 400	2.0 g
Polyvinylpyrrolidone 360 K	2.0 g
Bovine serum albumin	2.0 g
make up in 3 x SSC and sterilize by filtration through a 0.22 μ m Millex GV low binding filter, store in aliquots at -20 °C	

NYPHY: (100 ml)

Formamide	45.0 ml
100 x Denhardt's	7.5 ml
20 x SSPE	25.0 ml
25% SDS	0.4 ml
6.2 mg/ml calf thymus DNA, denatured	8.2 ml
sterile bidest water	13.9 ml

NYHY: (100 ml)

Formamide	45.0 ml
100 x Denhardt's	2.0 ml
6.2 mg/ml calf thymus DNA, denatured	9.0 ml
25% SDS	0.4 ml
20 x SSC	25.0 ml
sterile bidest water	18.6 ml

NCPHY: (100 ml)

Formamide	45.0 ml
20 x SSC	25.0 ml
100 x Denhardt's	5.0 ml
1 M NaPO ₄ pH 6.8	5.0 ml
25% SDS	0.4 ml
6.2 mg/ml calf thymus DNA, denatured	3.2 ml
sterile bidest water	16.4 ml

NCHY: (100 ml)

Formamide	45.0 ml
20 x SSC	25.0 ml
100 x Denhardt's	2.0 ml
1 M NaPO ₄ pH 6.8	2.0 ml
25% SDS	0.4 ml
6.2 mg/ml calf thymus DNA, denatured	3.2 ml
sterile bidest water	22.4 ml

TFB: (Hanahan 1983)

100 mM KCl
 45 mM $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$
 10 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$
 3 mM Hexamine cobalt trichloride
 made up in 20 mM K⁺MES (pH adjusted with KOH to 6.3), sterile filtered with a .22 μm filter and stored at 4 °C.

10 x Ligation buffer A: (Maniatis 1982)

660 mM Tris⁺Cl (pH 7.5)
 50 mM MgCl_2
 50 mM dithiothreitol (DTT)
 10 mM ATP

10 x Ligation buffer B: (Rusche 1985)

250 mM Tris⁺acetate (pH 7.5)
 50 mM MgCl_2
 5 mM dithiothreitol (DTT)
 1 mM ATP
 20 mM $((\text{NH}_3)_6\text{CoCl}_3)$

Bresa Buffer 1:

100 mM Tris⁺Cl pH 7.5
 1.0 M NaCl
 5 mM MgCl_2
 0.05% Triton X-100

Bresa Buffer 3:

100 mM Tris⁺Cl pH 9.5
 100 mM NaCl
 5 mM MgCl_2

20 x TAE for electroblotting:

200 mM Tris base
 100 mM Na acetate
 10 mM Na EDTA
 adjust to pH 7.8 with glacial acetic acid

10 x CIP buffer:

(Maniatis 1982)
 0.5 M Tris⁺Cl (pH 9.0)
 10 mM MgCl_2
 1 mM ZnCl_2
 10 mM spermidine

10 x CIP stop buffer:

(Colloquium 1984)
 100 mM Tris⁺Cl (pH 8.0)
 1 M NaCl
 100mM EGTA

10 x DNase buffer:

500 mM Na acetate (pH 6.5)
 100 mM MgCl_2
 20 mM CaCl_2

Bresa Buffer 2:

100 mM Tris⁺Cl pH 9.5
 1.0 M NaCl
 5 mM MgCl_2

Bresa Buffer 4:

10 mM Tris⁺Cl pH 7.5
 1 mM EDTA

TE: (Maniatis 1982)

10 mM Tris⁺Cl (pH 8.0)
 1 mM EDTA (pH 8.0)

2.2. Strains, plasmids and phages

2.2.1. *Escherichia coli* strains

Designation	Relevant markers	Reference
DH 1	F ⁻ , <i>gyrA</i> 96, <i>recA</i> 1, <i>endA</i> 1, <i>thi</i> 1, <i>hsdR</i> 17 (r ⁻ , m ⁺), <i>supE</i> 44	Hanahan, D. (1983)
GM 48	<i>dam</i> , <i>dcm</i>	Davison, J. (1984b)
71/18	Δ (<i>lac</i> - <i>pro</i>); F' (<i>lacI</i> ^q , <i>lacZ</i> Δ M15, <i>pro</i> ⁺ , <i>supE</i>)	Dente, L. (1983)
AMA 1004	<i>strA</i> ^r , <i>hsdR</i> ⁻ , <i>trpC</i> 9830, <i>leuB</i> 6, Δ (<i>lac IPOZ</i>) C 29, <i>lacY</i> ⁺ , <i>recA</i> ⁺	Casabadan, M. <i>et.al.</i> (1983)
KL 16-99	Hfr, <i>recA</i> 1, <i>str</i> ^S	Low, K.B. (1968)
PDC 7	same as AMA 1004, but <i>thyA</i>	this work
PDC 75	same as AMA 1004, but <i>recA</i> 1	this work

2.2.2. Plasmids and phages

Designation	Relevant Characteristics	Reference
YEp24	Ap ^r , Tc ^r , <i>URA</i> 3, 2 μ ori	Carlson, M. <i>et. al.</i> (1982)
pEMBL Y 23	Ap ^r , <i>lac POZ'</i> (<i>a</i> fragment), f1 ori, 2 μ ori, <i>URA</i> 3	Baldari, C. <i>et. al.</i> (1985)
pEMBL Y 31	Ap ^r , <i>lac POZ'</i> (<i>a</i> fragment), f1 ori, 2 μ ori, <i>LEU</i> 2	Baldari, C. <i>et.al.</i> (1985)
pDB 262	Tc ^{S/r} , λ cI <i>p</i> _{RM} , 2 μ ori, <i>LEU</i> 2	Beach, D. <i>et. al.</i> (1982b)
YEp Z36	Ap ^r , <i>lac Z</i> fusion vector, 2 μ ori, <i>LEU</i> 2	Oberto, J. <i>et. al.</i> (1985)
YEp Z100	Ap ^r , <i>lac Z</i> fusion vector, 2 μ ori, <i>LEU</i> 2	Oberto, J. <i>et. al.</i> (1985)
f1 IR1	derivative of f1, but resistance to negative interference of mini phages	Dotto, G.P. <i>et. al.</i> (1981)

2.2.3. *Saccharomyces cerevisiae* strains

Designation	Relevant markers	Source
DBY 918	<i>a</i> , <i>his</i> 4-38	D. Botstein
DBY 939	<i>a</i> , <i>suc</i> 2-215; <i>ade</i> 2	D. Botstein
GRF 18	<i>a</i> , <i>leu</i> 2-3, 2-112; <i>his</i> 3-11, 3-15; <i>CAD</i>	A. Hinnen
6657-9B	<i>a</i> , <i>leu</i> 2-3, 2-112; <i>his</i> 4Δ34; <i>CAD</i>	A.Hinnen
<i>a ura</i> 3 ws	<i>a</i> , <i>ura</i> 3, triple mutant	F. Lacroute
<i>a ura</i> 3	<i>a</i> , <i>ura</i> 3, triple mutant	F. Lacroute
z0 7	<i>a</i> , as GRF 18 but <i>cad</i> 1-7	this work
z0 42	<i>a</i> , as GRF 18 but <i>cad</i> 1-42	this work
z0 49	<i>a</i> , as GRF 18 but <i>cad</i> 1-49	this work
z0 88	<i>a</i> , as GRF 18 but <i>cad</i> 1-88	this work
z0 107	<i>a</i> , as GRF 18 but <i>cad</i> 1-107	this work
z0 124	<i>a</i> , as GRF 18 but <i>cad</i> 1-124	this work
z0 125	<i>a</i> , as GRF 18 but <i>cad</i> 1-125	this work
z0 132	<i>a</i> , as GRF 18 but <i>cad</i> 1-132	this work
PDY 100	<i>a</i> , <i>cad</i> 1-132, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 102	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 103	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 104	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 105	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 106	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 107	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 108	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 110	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 113	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 114	<i>a</i> , <i>cad</i> 1-107, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 115	<i>a</i> , <i>cad</i> 1-107, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 116	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 117	<i>a</i> , <i>cad</i> 1-88, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 118	<i>a</i> , <i>cad</i> 1-125, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 119	<i>a</i> , <i>cad</i> 1-125, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 120	<i>a</i> , <i>cad</i> 1-125, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 121	<i>a</i> , <i>cad</i> 1-125, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 123	<i>a</i> , <i>cad</i> 1-125, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 124	<i>a</i> , <i>cad</i> 1-107, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁺	this work
PDY 202	<i>a</i> , <i>cad</i> 1-125, <i>ura</i> ⁻ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 203	<i>a</i> , <i>cad</i> 1-125, <i>ura</i> ⁻ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 204	<i>a</i> , <i>cad</i> 1-132, <i>ura</i> ⁻ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 205	<i>a</i> , <i>cad</i> 1-132, <i>ura</i> ⁻ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 207	<i>a</i> , <i>cad</i> 1-88, <i>ura</i> ⁻ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work

continued.....

Designation	Relevant markers	Source
PDY 208	<i>a</i> , <i>cad</i> 1-88, <i>ura</i> ⁻ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 209	<i>a</i> , <i>cad</i> 1-124, <i>ura</i> ⁻ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 210	<i>a</i> , <i>cad</i> 1-7, <i>ura</i> ⁻ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 211	<i>a</i> , <i>cad</i> 1-7, <i>ura</i> ⁻ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 212	<i>a</i> , <i>cad</i> 1-7, <i>ura</i> ⁻ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 213	<i>a</i> , <i>cad</i> 1-7, <i>ura</i> ⁻ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 401	<i>a</i> , <i>cad</i> 1-107, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁻	this work
PDY 402	<i>a</i> , <i>cad</i> 1-132, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁻	this work
PDY 403	<i>a</i> , <i>cad</i> 1-88, <i>ura</i> ⁻ , <i>his</i> ⁺ , <i>leu</i> ⁻	this work
PDY 500	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 501	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 502	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 506	<i>a</i> , <i>cad</i> 1-107, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 507	<i>a</i> , <i>cad</i> 1-107, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 508	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 510	<i>a</i> , <i>cad</i> 1-107, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 512	<i>a</i> , <i>cad</i> 1-49, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 514	<i>a</i> , <i>cad</i> 1-125, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 515	<i>a</i> , <i>cad</i> 1-125, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 516	<i>a</i> , <i>cad</i> 1-88, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 517	<i>a</i> , <i>cad</i> 1-88, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 518	<i>a</i> , <i>cad</i> 1-7, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 519	<i>a</i> , <i>cad</i> 1-49, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁺	this work
PDY 600	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁻	this work
PDY 601	<i>a</i> , <i>cad</i> 1-7, <i>ura</i> ⁺ , <i>his</i> ⁻ , <i>leu</i> ⁻	this work
PDY 700	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁺ , <i>his</i> ⁺ , <i>leu</i> ⁻	this work
PDY 701	<i>a</i> , <i>cad</i> 1-42, <i>ura</i> ⁺ , <i>his</i> ⁺ , <i>leu</i> ⁻	this work
PDY 704	<i>a</i> , <i>cad</i> 1-107, <i>ura</i> ⁺ , <i>his</i> ⁺ , <i>leu</i> ⁻	this work
PDY 705	<i>a</i> , <i>cad</i> 1-107, <i>ura</i> ⁺ , <i>his</i> ⁺ , <i>leu</i> ⁻	this work
PDY 707	<i>a</i> , <i>cad</i> 1-125, <i>ura</i> ⁺ , <i>his</i> ⁺ , <i>leu</i> ⁻	this work
PDY 708	<i>a</i> , <i>cad</i> 1-88, <i>ura</i> ⁺ , <i>his</i> ⁺ , <i>leu</i> ⁻	this work
PDY 710	<i>a</i> , <i>cad</i> 1-88, <i>ura</i> ⁺ , <i>his</i> ⁺ , <i>leu</i> ⁻	this work
PDY 711	<i>a</i> , <i>cad</i> 1-7, <i>ura</i> ⁺ , <i>his</i> ⁺ , <i>leu</i> ⁻	this work

2.2.4. Other strains

Organism	Relevant Markers	Source
<i>Schizosaccharomyces pombe</i>	<i>h</i> ⁺ , <i>leu</i> 1-32	P. Munz
<i>Schizosaccharomyces pombe</i>	<i>h</i> ⁻ , <i>leu</i> 1-32	P. Munz
<i>Fusarium oxysporum</i>	CAD	R. Megnet

2.3. Chemicals and enzymes

All chemicals were at least of analytical grade. They were purchased from one of the following manufacturers: Sigma, Merck, BRL, Bresa, Fluka, Serva, Eastman Kodak, Seakem, BioRad, Calbiochem. All solutions were made in water of reagent grade. Chemicals were generally not further purified or treated specially, with the following exceptions:

DMSO for *E. coli* transformations: The purest grade available commercially (Fluka) was aliquoted into .5 ml portions in an anaerobic hood and stored in liquid nitrogen.

Hexane for extraction in CAD assays: Analytical grade hexane was further purified (Perrin 1980) by shaking 1 l three times with 250 ml of nitrating acid (a mixture of 50% v/v of sulphuric acid and nitric acid), followed by three washes with distilled water and three washes with .1 N NaOH. Hexane was subsequently dried over sodium lead alloy, distilled from sodium and stored cool and dark. Immediately prior to use, hexane was then passed over a column (50 x 2.5 cm) packed with basic alumina.

Pentane for extraction in CAAD assay: Analytical grade pentane was further purified just before use by passing over a column (50 x 2.5 cm) packed with basic alumina.

Diethylether for extraction in CAAD assay: Analytical grade diethylether was distilled from sodium and stored cool and dark.

Formamide for nucleic acid hybridizations: 1 l of analytical grade formamide with an initial conductivity of 470 $\mu\text{mho}_{20}^{\circ}\text{C}$ was recrystallized for 48 h at 0 $^{\circ}\text{C}$ with slow stirring after which the conductivity fell to 150 $\mu\text{mho}_{20}^{\circ}\text{C}$. This material was then stirred for 6 hrs with a mixture of cation and anion exchange resin. This material was then passed over a column (75 x 1.5 cm) packed with 15 g of mixed bed resin (AG 501-X8, 20-50 mesh) (Ofengand 1980), after which the conductivity was 7 $\mu\text{mho}_{20}^{\circ}\text{C}$. Deionized formamide was stored in aliquots at -70 $^{\circ}\text{C}$.

Phenol for nucleic acid extractions: Analytical grade phenol was further purified by distillation under reduced pressure with an inert atmosphere (either N_2 or He). This material was equilibrated with .1 M Tris $\cdot$ Cl pH 8 and stored in aliquots at -20 $^{\circ}\text{C}$. "Phenol" for extractions is this material with the addition of 0.1% hydroxyquinoline and 0.2% β -mercaptoethanol.

Disposal of hazardous chemicals: Phenol, chloroform, hexane, pentane, diethylether, as well as solutions containing ethidium bromide were collected for disposal according to federal laws. Mobile phases containing methanol were collected for reprocessing. Formamide was hydrolyzed by the addition of dilute sulfuric acid. Solutions containing PMSF or labware contaminated with PMSF were soaked one week in 3 M NaOH prior to disposal. Solutions contaminated with 1-methyl-3-nitro-1-nitrosoguanidine (MNNG) were slowly added to HBr in glacial acetic acid, and soaked for 1 week prior to disposal.

Restriction enzymes were purchased from BRL, Boehringer Mannheim and New England Biolabs. DNA Ligase was from BRL, calf intestinal alkaline phosphatase (CIP) was from Boehringer Mannheim. Ribonuclease A and ribonuclease T_1 were obtained from Serva and prepared as described (Maniatis 1982).

Novozym 234 was a gift of Novo Industri, Copenhagen, Denmark. Zymolyase 5000 and Zymolyase 60000, produced by Seikagaku Kogyo Co., were purchased from Miles Laboratories. Lysozyme was from Sigma, proteinase K was from Merck.

2.4. Instruments

Microscopes: Zeiss, Wetzlar, FRG

Incubators: Modell 3956 Incubator; Forma Scientific, Marietta, OH, USA

Rotary shakers: Modell HT; Infors AG, Basel, Switzerland

Freeze-dryer: Modulyo; Edwards, Crawley, England

Rotary evaporator: Rotavapor RT 110; Büchi, Flawil, Switzerland

Analytical balance: Sartorius, Göttingen, FRG

Spectrophotometer: Beckman DU-5; Beckman, Fullerton, CA, USA

Transilluminator: Fotodyne UV System Model 3-4125; Fotodyne, New Berlin, WI, USA

Documentation: Polaroid MP-4 camera and 667 films; Polaroid, Cambridge, MA, USA

Centrifuges: Speed-Vac Concentrator; Savant, Hicksville, NY, USA. Eppendorf model 3200; Netheler + Hinz, Hamburg, FRG. TJ-6 centrifuge with TJR refrigeration unit; J-21B with rotors JA 10 and JA 20; L8-70 with rotors SW 41 Ti, VTi 50 and Ti 50; all Beckman, Fullerton, CA, USA. MSE High Speed 18 with the 16 x 15ml rotor; MSE Scientific Instruments, Crawley, England.

Hoods: Gelaire Class 100 Laminar Air Flow and Biohazard type BHNG; Flow Laboratories, Meckenheim, FRG

HPLC: Automated Gradient Controller, High Pressure Pumps M 45 and 6000 A, WISP 710 B, M 490 Programmable Multiwavelength Detector, M 730 Data Module, Column Switching Valve; all from Waters Instruments, Milford, MA, USA

Waterbaths: W19 waterbath with thermostat type 000-4526; Haake, Karlsruhe, FRG

NMR: Bruker WP 80; Bruker, Karlsruhe, FRG

Sonifier: Branson Sonifier Cell Disruptor B 15 with microtip; Branson Sonic Power Co, Danbury, CN, USA

Elution chamber: Biotrap; Schleicher & Schuell, Dassel, FRG

Power Supplies: Buchler 3-1500 Constant Power Supply; Buchler, Fort Lee, NJ, USA. Model 100 Power Supply; BRL, Gaithersburg, MD, USA

Electrophoresis apparatus: Horizontal Model H 3; BRL, Gaithersburg, MD, USA. Self-constructed models for horizontal agarose electrophoresis as well as a self-constructed model for electroelution and electroblotting

Densitometer: Chromoscan; Joyce Loeb Ltd., Gateshead 11, England

Optical filters: OG 590 and BG 38; Schott, Mainz, FRG

2.5. General methods

Sterilization:

All solutions were sterilized by autoclaving (20 min at 121 °C) with the following exceptions, which were sterilized by filtration through .22 μ m filters: 25% SDS; Denhardt's solution and 5% BSA (with low binding filters); PEG solutions for transformation; vitamin, tryptophan and casamino acid solutions. All glassware was sterilized by dry heat (6 h at 180 °C). All Eppendorf caps and centrifuge tubes used were sterile.

Precipitation of nucleic acids:

Nucleic acids were precipitated by the addition of either half a volume of 7.5 M ammonium acetate or 0.1 volume of 4 M LiCl (Colloquium 1984), followed by the addition of either 0.8 volumes of *i*-propanol or 2.5 volumes of 96% ethanol. The samples were mixed and allowed to stand either on ice for 20 min (Zeugin 1985) or at -20 °C for 30 min. Material in small-scale reactions was recovered by centrifugation at 12 krpm in an Eppendorf centrifuge for 10 min; large-scale reactions were centrifuged at 4 °C, 14 krpm in the JA 20 rotor. Pellets were washed in 80% ethanol and briefly dried in a speed-vac centrifuge or in a freeze-dryer.

Preservation of strains:

E. coli strains were preserved by adding 1 vol sterile 87% glycerol to a fresh overnight culture in an Eppendorf cap. This was mixed thoroughly and then stored either at -20 °C or in liquid nitrogen. Yeast strains were stored by resuspending a pellet of a fresh overnight culture in .25 volumes of sterile 5% skim milk and dropping this suspension slowly onto heat sterilized silica gel in 1 dram vials. These were then stored for a week in a desiccator with the caps only loosely screwed on. The caps were then fastened tightly and stored at 4 °C. Alternatively, fresh overnight cultures were mixed with an equal volume of sterile 87% glycerol, mixed well and plunged into liquid nitrogen. Phages were stored in TE at -20 °C.

Determination of dry weight:

Dry weight was determined by pelleting cells in weighed 2.2 ml Eppendorf caps, discarding the supernatant and freeze-drying the biological material.

All other general techniques were as described by Maniatis (1982) or by Sherman (1983).

2.6. Preparation of plasmid DNA

Plasmid DNA from *E. coli* was prepared as described in the two following sections. It was generally not further purified by isopycnic centrifugation.

2.6.1. Lysis by alkali

The following method was based on the methods of Birnboim (1979) and Wackernagel (personal communication) and was modified as follows:

1. Cells from fresh overnight cultures were resuspended in one half volume of solution I, without lysozyme; mixed gently but thoroughly until the suspension was homogeneous, followed by the addition of the other half of solution I containing all ly-

sozyme, and placed on ice for 45 min.

2. Solution II was added, the contents of the cap mixed gently by inversion until the suspension cleared; if it did not clear after 5 min, more SDS was added. After clearing the cap was put on ice for 10 min until the SDS - protein complexes began to precipitate.

3. Solution III was added, the cap mixed by inversion and put on ice for 45 min. The denatured material was pelleted, the supernatant transferred to a new cap or tube and precipitated with *i*-propanol at 0 °C

4. The pellet was resuspended in TE, (for volumes, see table below). In a small-scale preparation, I proceeded to step 5. In a large-scale preparation, .05 volume of .5 M EDTA and .02 volume of RNase A, prepared as described by Maniatis (1982), were added and the material incubated for 45 min at 37 °C.

5. .1 volume 4 M LiCl were added, mixed, then 1 volume of phenol and chloroform/*i*-amyl alcohol each Maniatis (1982) were added and the preparation extracted by vigorous vortexing. Phases were separated by centrifugation, the aqueous phase transferred to a new cap or tube and the extraction repeated once. Extraction was repeated once more with chloroform only.

6. 2.5 volumes of ethanol were added for precipitation, after centrifugation the pellet was washed with 80% ethanol and dried. The pellet was redissolved in 1 volume water, reprecipitated with ammonium acetate and ethanol, dissolved in TE (small-scale in 100 µl, large-scale in 1 ml for concentration measurement) and stored at -20 °C.

The material from a 1 ml overnight culture was sufficient for at least 50 analytical procedures.

Solution I	Solution II	Solution III
50 mM Glucose	0.2 N NaOH	3 M Na ⁺ acetate/
10 mM CDTA	1% SDS	formiate pH 4.8
25 mM Tris·Cl pH 8		
2 mg/ml lysozyme final concentration.		

Culture volume	I	II	III	TE
1.0 ml	150.0 µl	240.0 µl	150.0 µl	100.0 µl
10.0 ml	700.0 µl	1120.0 µl	700.0 µl	1.0 ml
40.0 ml	4.0 ml	6.5 ml	4.0 ml	4.0 ml
1000.0 ml	20.0 ml	32.0 ml	20.0 ml	5.0 ml

2.6.2 Lysis by boiling

The following procedure was modified after Holmes (1981):

1. The bacteria from a fresh overnight culture (1 ml) were pelleted in an Eppendorf cap by a low speed centrifugation, the supernatant was removed by aspiration until the pellet was dry.

2. The pellet was resuspended in 350 µl of solution I (see below); the caps were then

placed on ice for 10 min with the lid open.

3. 50 μ l of solution II were added, the lids shut and the caps vortexed briefly, taking care that they stayed cold, then immersed in a boiling water bath for precisely 1 min. This and the following steps of the procedure up to step 5 were performed in lots of 12 only, which was essential for a good yield and quality of the preparation.

4. Immediately after boiling them, the caps were centrifuged at the highest speed possible in an Eppendorf centrifuge for 20 min, the slimy pellet was removed with a toothpick and discarded.

5. 100 μ l 7.5 M ammonium acetate were added, mixed, 500 μ l *i*-propanol added, mixed and put on ice for 30 min.

6. Nucleic acids were pelleted by spinning for 10 min, the pellet was washed with 80% ethanol, dried, resuspended in 100 μ l water, 50 μ l 7.5 M ammonium acetate and 300 μ l 96% ethanol were added, put on ice 30 min, spinned for 10 min, the pellet dried and dissolved in 50 μ l TE; and stored at -20°C

Solution I:

8.0% Saccharose

0.5% Triton X-100

25.0 mM CDTA

20.0 mM Tris·Cl pH 8

Solution II:

5.0 mg/ml lysozyme in

10 mM Tris·Cl pH 8

2.6.3. Plasmid rescue from *S. cerevisiae*

1. Transformed yeast cells (20 ml) were recovered from a fresh culture in selective media by centrifugation, then washed twice in 0.9 M sorbitol, 25 mM EDTA followed by resuspension in 10 ml of the preparative digestion buffer (see: 2.8.1). This suspension was gently shaken at 30°C for 1 h. Incubation was briefly prolonged if protoplastation was incomplete.

2. Protoplasts were pelleted at 2 krpm (JA 20 rotor), the supernatant removed with a pipette. The protoplasts were washed very carefully once in 0.9 M sorbitol, 25 mM EDTA, harvested at 2 krpm and gently resuspended in 500 μ l 0.6 M sorbitol, 25 mM EDTA.

At this stage, the protoplasts were lysed either by a modification of the alkaline lysis procedure for *E. coli* (Birnboim 1979) or by a modification of an acid phenol technique (Kieser 1984).

Alkaline lysis:

1. The protoplast suspension was lysed by adding 800 μ l of 0.2 N NaOH, 2% SDS. The suspension was mixed gently by inversion until it cleared, then placed on ice for 10 min.

2. Denatured material was precipitated by adding 500 μ l of solution III (see 2.6.1.), mixing the caps by inversion. The caps were placed on ice for 45 min, spinned for 10 min; the supernatant (approximately 1.6 ml) divided between 2 Eppendorf caps. The nucleic acids were precipitated by adding 600 μ l *i*-propanol.

3. This material was then extracted and reprecipitated from water / ammonium acetate before transforming *E. coli*.

Acid phenol:

1. The protoplast suspension was lysed by the addition of 250 μ l 0.3 N NaOH, 2% SDS, followed by immediate vigorous vortexing and subsequent incubation at 55 °C for 30 min.
2. 80 μ l of acid phenol (5 g reagent grade but undistilled phenol, 5 ml chloroform, 1 ml bidest H₂O and 5 mg 8-hydroxyquinoline) were added and the cap immediately vortexed for 3 min. After centrifugation the upper phase was removed and nucleic acids precipitated by LiCl and *i*-propanol.
3. This material was reprecipitated from water / ammonium acetate before transforming *E. coli*.

2.6.4. Plasmid purification by isopycnic centrifugation

Plasmids were purified in isopycnic CsCl gradients by a modified rapid method (Garger 1983). The DNA to be purified was extracted from a 500 ml overnight culture by alkaline lysis (volumes for the solutions same as for 1 l), followed by RNase A digestion and two phenol extractions. After precipitation the material was dissolved in 1 ml of sterile bidest water.

1. The volume of this solution was adjusted to exactly 1.85 ml with water, followed by the addition of 3.07 g CsCl. This mixture was allowed to dissolve at 37 °C, then 330 μ l of a filtered 10 mg/ml ethidium bromide solution were added. This solution then had a density of $\rho = 1.75$ and a volume of 3 ml.
2. Approximately 7 ml of the upper phase ($\rho = 1.47$), made up by dissolving 5.116 g CsCl in 6.644 ml TE, were filled into a polycarbonate tube (Beckman # 339574). The denser phase containing the nucleic acid was then carefully layered beneath this phase with a long Pasteur pipette, taking care not to disturb the solution interface. Opposing tubes were balanced to 10 mg with the upper phase solution.
3. Centrifugation was for 12 h at 40 krpm, 20 °C in an Ti 50 rotor. The run was terminated with brakes off. Bands visible in UV light were collected from above with a Pasteur pipette. Ethidium bromide was removed by extracting five times with three volumes of water-saturated *n*-butanol; CsCl was removed by subsequent dialysis against water (30 min) and three times against 1 l TE. Plasmid DNA was reprecipitated with ammonium acetate and ethanol and stored in TE at -20 °C. Yields were usually between .5 to 2 mg.

2.7. Preparation of single – stranded DNA

Single-stranded DNA was prepared from transformed *E. coli* cells by superinfection with coliphage ϕ 1 IR1 by a modification of the procedure described by Baldari (1985) and Dente (1983).

1. 20 ml of pre-warmed SOB with 100 μ g/ml ampicillin was inoculated with 200 μ l from a fresh overnight culture of *E. coli* harboring plasmids with ϕ 1 replication ori-

gins and slowly rotated in a shaker at 37 °C. At an O.D.₆₀₀ of 0.3 the cells were infected with phage at a m.o.i. of 10. After 60 min the shaker was adjusted to 150 rpm, the cells were incubated for a further 5 hrs.

2. The cells were pelleted at room temperature, the supernatant was slowly added to 0.4 volumes of 2.5 M NaCl, 20% PEG 6000 in a JA 20 tube. After 30 min at room temperature, single-stranded packaged DNA was collected by spinning at 14 krpm. This pellet was resuspended in 800 μ l TE and reprecipitated. This material was suspended in 200 μ l TE and after the addition of 50 μ l of 2 mg/ml proteinase K, 1% SDS in TE, incubated overnight at 50 °C.

3. This material was extracted 3x with phenol / chloroform and reprecipitated twice with ammonium acetate and ethanol, finally resuspended in 200 μ l TE and stored at -20 °C.

2.8. Preparation of chromosomal DNA

2.8.1. *S. cerevisiae* high molecular weight DNA

High molecular weight DNA from yeast was prepared by a modification of the procedures of Beggs (1978) and Hinnen (1978).

1. 1 l of YEX were inoculated with 1 ml of a fresh stationary phase culture grown on YG. Cells were harvested at a titer of approximately 2×10^8 N/ml.

2. The cells were washed twice with 0.5 volume ice-cold sterile water followed by a 30 min incubation in 500 ml bidest H₂O, 1 ml β -mercaptoethanol at 30 °C. The cells were then resuspended in 150 ml of the following digestion buffer: 54 ml 2.5 M sorbitol, 15 ml 0.5 M KPO₄ (pH 7.5), 25 ml 0.5 M EDTA, 1 ml β -mercaptoethanol, 55 ml H₂O and 7.5 mg Zymolyase 60K and shaken slowly at 30 °C for 90 min.

3. When protoplastation was complete, the cells were washed once in 0.9 M sorbitol and then gently but very thoroughly resuspended in 30 ml of 60 mM EDTA. This thick suspension was added very slowly with a wide bore pipette to 60 ml of the following solution, preheated to 50 °C: 3.2 ml 25% SDS, 5 ml 0.5 M EDTA, 30 ml sterile H₂O and 10 mg proteinase K and incubated for a further 6 hrs.

4. To the lysed cells, the following solutions were added: 12 ml 3 M Na acetate (pH 6), 50 ml phenol, 50 ml chloroform with 4% *i*-amyl alcohol and extracted over night at 5 rpm in a tumbler. After centrifugation, the aqueous phase was recovered and re-extracted with phenol / chloroform. The nucleic acids were concentrated by extracting three times with unsaturated *n*-butanol where upon the volume was reduced to approximately 50 ml. This material was dialyzed extensively against TE and concentrated twice more with *n*-butanol. After dialysis against TE the volume was adjusted to 20 ml and 23.426 g CsCl were added and dissolved (ρ = 1.65). This solution was centrifuged in cellulose nitrate tubes in a SW 41 Ti rotor at 20 °C at 19.5 krpm for 168 hrs to pellet contaminating RNA. The supernatant was dialyzed against TE, and stored either at 4 °C over a drop of chloroform or as ethanolic precipitate at -20 °C.

2.8.2. *Fusarium* spp. DNA

High molecular weight chromosomal DNA from *Fusarium* spp. was prepared by a modification of the procedure of Raeder (1984).

1. 2 l of Fries medium was inoculated with 20 ml from an overnight culture grown in the same medium and aerated well at 30 °C. After approximately 18 h, when vegetative growth was maximal and only few if any spores had developed, the mycelia were harvested and washed with a total volume of 200 ml 100 mM EDTA, frozen in liquid nitrogen and freeze-dried.
2. The dry material was pulverised by grinding in a mortar followed by suspension in 80 ml of the following buffer: 50 mM Tris·Cl pH 8, 100 mM EDTA pH 8, 150 mM NaCl, 2% SDS, 20% toluene. This emulsion was shaken slowly at 30 °C in a rotary shaker for 48 hrs after which 50 ml of phenol / chloroform each were added and extracted over night at 5 rpm on a tumbler.
3. The aqueous supernatant was recovered after centrifugation and reextracted once with chloroform and twice with *n*-butanol. The nucleic acids were precipitated with *i*-propanol. The pellet was dissolved in 5 ml TE, the volume adjusted to 10 ml and 10.478 g solid CsCl were added to give a density of ($\rho = 1.6$).
4. The nucleic acids were centrifuged at 18 °C, 20 krpm for one week after which the chromosomal DNA was recovered from the supernatant as a distinct opaque band and dialyzed three times against 1 l TE. The high molecular weight chromosomal DNA was reprecipitated once from ammonium acetate / water with ethanol and stored as an ethanolic precipitate at -20 °C.

2.9. Preparation of total RNA

2.9.1. Preparation of RNA from *S. cerevisiae*

Total RNA from *S. cerevisiae* (total RNA from *S. pombe* was prepared identically) was prepared by a modification of the methods of McNeil (1986), Jensen (1983) and Kurtz (1984).

1. Cells were grown, if necessary in selective medium, to late exponential phase in a volume of 1 l, harvested and washed twice with ice-cold sterile water. They were re-suspended in 50 ml H₂O and 0.5 ml β -mercaptoethanol, and incubated at 30 °C for 30 min. After centrifugation, the pellet was resuspended in 10 ml cracking buffer (100 mM Tris·Cl pH 7.5, 100 mM LiCl, 20 mM β -mercaptoethanol) after which the suspension was frozen in an X-Press at -35 °C.
2. After 1 h the cells were fractured by 10 passages through the press. After thawing, 80 ml cracking buffer, 5 spoonfuls of sterile acid-washed glassbeads (mean diameter 0.5 mm), 50 ml each of phenol and chloroform with 4% *i*-amyl alcohol and 10 ml 25% SDS were added to the slurry. The material was then vortexed at the highest possible speed for 10 min. After the addition of 20 ml 100 mM CaCl₂ to precipitate surplus SDS, the phases were separated by centrifugation.
3. The nucleic acids were precipitated by adding 0.05 vol 4 M LiCl and 1 vol *i*-propanol. The pellet was dissolved in 30 ml water and spinned to remove insoluble ma-

terial. RNA was precipitated from the supernatant by the addition of an equal volume of 5 M LiCl, 50 mM Tris·Cl pH 7.5, followed by an incubation on ice for 30 min and collected by centrifugation. The pellet was resuspended in 20 ml H₂O, insolubles removed, and RNA reprecipitated.

4. This material was resuspended in 10 ml H₂O, an aliquot of 1 or 2 ml removed and the remainder stored at -70 °C. The following were added to the nucleic acid: Water, to give a final volume of 3 ml; then 2 mg proteinase K, 200 µl TE x 10, 40 µl 1 M Tris·Cl pH 8 and 100 µl 25% SDS were added and the solution incubated 1 h at 50 °C. Then, after the addition of 2.5 ml H₂O and 0.5 ml 4 M LiCl, the sample was extracted twice with phenol / chloroform followed by precipitation of the nucleic acids with ethanol.

5. The RNA was dissolved in 1 ml H₂O and an aliquot of 0.75 ml was removed and stored at -70 °C. 25 µl DNase buffer x 10 and 100 units of RNase free DNase were added to the remaining 250 µl and the residual DNA degraded for 45 min at 37 °C followed by the addition of 0.1 vol 4 M LiCl, two phenol / chloroform extractions and precipitation by ethanol. The pellet was dissolved in 250 µl H₂O and stored at -70 °C.

2.9.2. Preparation of total RNA from *Fusarium* spp.

Total RNA from *Fusarium* spp. was prepared by dissolving the RNA pellet from step 4 of 2.8.2. in 10 ml H₂O after which the protocol for yeast total RNA preparations was followed, starting at step 4.

2.10. Restriction analysis

Restriction analysis was performed as described in Maniatis (1982). Generally, the reactions were performed in the buffers suggested by the manufacturers. For analytical purposes, the material was overdigested two-fold, for preparatory purposes the material was overdigested five-fold. Digested material was generally not extracted before electrophoretical analysis.

2.11. Gel electrophoresis

2.11.1. General conditions for non - denaturing gels

Gel electrophoresis was performed on horizontal agarose gels as described by Maniatis (1982). TBE, TBEE, TPE and TPEE buffers were as described by Maniatis (1982), the suffix "E" denotes the addition of ethidium bromide to 250 ng/ml final concentration. Gels were prepared by melting the desired amount of agarose in the desired volume of electrophoresis buffer in an autoclave at 100 °C, cooling the gel to 55 °C prior to pouring it. Except when stated explicitly all separations were performed at 4 °C in buffers with ethidium bromide with voltage gradients of 2 V/cm for fragments smaller than 20 kbp (Aaij 1972, Johnson 1977 and McDonell 1977) and gradients of 0.5 V/cm or 0.25 V/cm for larger fragments or species (Fangman 1978). Gels were not destained prior to photography. Samples were mixed with slotmarker II (Maniatis 1982) before applying them to the wells. The molecular weight of the samp-

les applied was estimated from a comparison of their migration distances with the migration distances of molecules of known size (Lumpkin 1982).

2.11.2. Size markers

DNA species	Size (bp)
<i>Hind</i> III fragments of λ :	23130.
(Sanger 1982)	9416.
	6682.
	4361.
	2322.
	2027.
	564.
<i>Eco</i> R I fragments of pCB 1 - 6:	8802.
(Childs 1984)	8062.
	7322.
	6582.
	5842.
	5102.
<i>Eco</i> R I / <i>Pvu</i> II digestion of pCB 1 - 6:	6507.
(Childs 1984)	5767.
	5027.
	4287.
	3547.
	2807.
	2295.
<i>Eco</i> R I / <i>Sal</i> I digestion of pCB 1 - 6:	5092.
(Childs 1984)	4352.
	3712.
	3612.
	2872.
	2132.
	1392.
<i>Bam</i> H I / <i>Pst</i> I / <i>Pvu</i> II of pBR 322:	1693.
(Sutcliffe 1979) and (Peden 1983)	1545.
	1125.
<i>Bam</i> H I / <i>Pst</i> I / <i>Pvu</i> II / <i>Eco</i> R I of pBR 322:	1693.
(Sutcliffe 1979) and (Peden 1983)	1545.
	750.
	375.

2.11.3. Denaturing electrophoresis for RNA

Separations of total RNA prior to northern transfer and hybridization were performed on horizontal denaturing formaldehyde gels as described by Lehrach (1977) and Maniatis (1982) with the following modifications:

1. 960 mg agarose were mixed with 68.5 ml H₂O, melted in an autoclave at 100 °C and cooled to 60 °C after which 4 ml of running buffer x 20 (1 M HEPES pH 7.8, 10 mM EDTA) and 16 ml of 12.2 M formaldehyde were added. The gel was poured immediately afterwards and submerged after setting.
2. The samples were prepared by lyophilization of the amounts to be analyzed, followed by resuspension in: 2 µl running buffer x 20, 7 µl 12.2 M formaldehyde, 20 µl purified formamide and 7.5 µl H₂O. The samples (and DNA restriction fragments as size markers, labelled with Photobiotin) were then denatured by heating to 55 °C for 15 min; followed by cooling to room temperature after which 4 µl of RNA slot marker (Maniatis 1982) were added. After mixing, the samples were applied to the gel, electrophoresis conditions were: 30 min at 1 V/cm, then 2.5 V/cm until the tracking dye was 1 cm from the edge of the gel.

2.12. Isolation of DNA fragments

Restriction fragments were isolated either by electrophoretically driving the desired fragment separated before on an agarose gel onto DEAE paper (NA 45, Schleicher & Schuell), by excising a band from a gel and electroeluting it into the trap chamber of Biotrap (Schleicher & Schuell) or by trapping the fragment in a high-salt cushion (7.5 M ammonium acetate) in an electroelution apparatus after excising it from an agarose gel. For the former two procedures, the manufacturer's suggestions were followed, conditions for the latter one were: separation buffer was degassed TBE and the voltage applied was 50 V for 30 min, then 100 V for a further hour.

2.13. Ligation of DNA fragments

Ligations were performed either as described by Maniatis (1982) and Dugaiczky (1975) in ligation buffer A or as described by Rusche (1985) and Legerski (1985) in ligation buffer B, with modifications in the reaction conditions in the latter procedure. The ratios of insert to vector DNA were exactly as described by Legerski (1985), however, the joining reaction was performed as follows:

DNA species, 10 x ligation buffer B and, if necessary, water were added to a total volume of 20 µl in 1 x ligation buffer B and incubated 5 min at 23 °C. Then 1 U T4 DNA ligase were added and the incubation continued for 30 min at 23 °C, after which KCl was added to a final concentration of 30 mM. The reaction was continued for a further 2 hrs at 23 °C after which the mixture was extracted with phenol / chloroform, precipitated and dissolved in 20 µl TE prior to transformation.

2.14. Transformations

2.14.1. Transformation of *E. coli*

Transformations of *E. coli* were performed exactly as described by Hanahan (1983)

with TFB buffer. Manipulations were performed in 2.2 ml Eppendorf caps and heat shock was for 90 s at 42 °C. Expression times before plating were 1 h.

2.14.2. Transformation of *S. cerevisiae*

Transformations of yeast were performed by a modification of the methods of Beggs (1978), Hinnen (1978), Struhl (1979) and Gerbaud (1979).

1. Strains to be transformed were grown to saturation on YG for 48 hrs with vigorous aeration. Exponentially growing cells from 100 ml YEX, supplemented with either uracil or leucine and histidine as suggested by Sherman (1983), inoculated with 0.005 volumes of the YG preculture were harvested at a density of 4×10^7 N/ml. They were washed twice with sterile ice-cold water and collected by centrifugation 4 krpm, 4 °C, 10 min in sterile JA 10 tubes.

2. Cells were then washed once in 1 volume 0.9 M sorbitol, 25 mM EDTA, pelleted and resuspended thoroughly in 25 ml of the following buffer: 0.8 M sorbitol, 83 mM KPO_4 (pH 7.5), 83 mM EDTA and 0.1 mg/ml Zymolyase; after which the suspension was incubated for 1 h at 30 °C in a rotary shaker at 30 rpm. After protoplastation was complete, the suspension was transferred to JA 20 tubes and the cells collected at 1 krpm, 4 °C, 12 min.

3. The protoplasts were carefully washed twice in 35 ml each of the following buffer: 0.9 M sorbitol, 10 mM Tris·Cl pH 7.5, 10 mM CaCl_2 and collected as above. The supernatant was decanted and the final pellet was resuspended in 2 ml of the same buffer to give a protoplast density of approximately 2×10^9 N/ml.

4. The protoplasts to be transformed were dispensed in 100 μl aliquots in 1.5 ml Eppendorf caps to which plasmid DNA was added in less than 10 μl . This suspension was mixed gently and incubated at room temperature for 20 min after which 1 ml of: 20% PEG 4000, 10 mM CaCl_2 , 20 mM Tris·Cl pH 7.5 (sterile filtered) were added, mixed by inversion and further incubated for 20 min at room temperature. The protoplasts were collected by centrifugation at low speed and resuspended in 1 ml regeneration mix (see below) and incubated for 1 h at 30 °C. Protoplasts were then pelleted and resuspended in 250 μl fresh regeneration mix after which they were added to 10 ml molten (55 °C) top agar (same composition as YMCS - x but 2% agar) and immediately poured on prewarmed (37 °C) selective plates (YMCS - x). After setting, the plates were inverted and incubated at 30 °C.

Regeneration mix:

Yeast extract	1.6 g
bidest H ₂ O	66.0 ml
autoclave, then add the following:	
2.5 M Sorbitol	48.0 ml
0.2 g/ml KH ₂ PO ₄	400.0 μ l
2 M MgSO ₄	100.0 μ l
100 mM CaCl ₂	8.0 ml
2 M Glucose	4.0 ml
also add uracil, leucine and histidine to the amounts suggested by Sherman (1983); if selection is for growth independence of one of these, reduce the amount added to 5% of the original amount.	

2.14.3. Transformation of *S. pombe*

S. pombe was transformed as described by Beach (1982a) with the exception that no salmon sperm carrier DNA was added.

2.15. Generation of *in-vivo lac* fusions.

In-vivo lac fusions were generated by a modification of the protocol suggested by Rose (1983).

1. Fresh overnight cultures of *E. coli* AMA 1004 transformed with plasmids containing sequences to be fused and grown under selective pressure in LB were used to inoculate 12 independent cultures. When these had grown to saturation the cells were pelleted and washed twice with minimal medium without carbon source and resuspended in one tenth of the original volume. The cells were then spread on M 10 plates with 0.2% lactose, 50 μ g/ml ampicillin and supplemented with leucine and tryptophan. The cells were incubated at 37 °C for periods up to three weeks during which colonies acquiring proficiency in lactose utilization continuously arose after a lag period of 3 to 4 days.

2. These clones were purified by streaking on selective plates after which they were grown up on rich liquid medium with 50 μ g/ml ampicillin for selection and lysed for plasmid analysis. Promising candidate *lac* fusions were further purified by retransforming the plasmid isolates into *E. coli*, plating them onto media selective for lactose utilization, which ensured a homogeneous plasmid population.

2.16. Analysis by Southern hybridization

Homologies between DNA sequences were analyzed by Southern hybridization (Southern 1975 and Maniatis 1982). Nitrocellulose filters with bound *S.cerevisiae* chromosomes separated by orthogonal field alternation gel electrophoresis (OFAGE) were a gift of Ms. Eva - Maria Geigl of gsf, Institut für Strahlenbiologie, Ingolstädter Landstraße 1, D - 8042 Neuherberg.

2.16.1. Preparation of probe DNA

1. Probe DNA was made free of RNA by chromatography on NACS prepac mini

columns (BRL); approximately 50 μg of DNA, dissolved in maximally 100 μl were added to 2 ml of equilibration and binding buffer (0.5 M NaCl in TE) and applied to the column after it had been hydrated. The column was washed with 10 volumes binding buffer by gravity flow, after which the bound species were eluted by applying elution buffer (0.75 M NaCl in TE) as described by BRL. The scrubbed DNA was recovered by precipitation and resuspended in water after which it was digested by restriction enzyme to obtain the desired fragments. This was followed by a phenol / chloroform extraction and reprecipitation after which the DNA was resuspended in water and its concentration determined photometrically.

2. Probe DNA was labelled by the photoaffinity label Photobiotin as described by Forster (1985) and the BRESA Labelling and Detection Kit instruction manual: DNA at 1 $\mu\text{g}/\mu\text{l}$ was mixed with an equal volume Photobiotin at 1 $\mu\text{g}/\mu\text{l}$ in an Eppendorf cap in the dark and put on ice for 10 min. The samples were kept on ice, irradiated with open lids for 30 min at a distance of 10 cm beneath an OSRAM HQL-R 400 watt lamp. A BG 38 glass filter was placed between the caps and the lamps to minimize damage to the probes by UV and IR irradiation.

3. All subsequent steps, carried out in normal light, were as described in the instruction manual. Quantitative precipitation of labelled DNA was achieved by adding 30 μg of tRNA to each cap prior to ethanol precipitation. Labelled probes were dissolved at 100 $\text{ng}/\mu\text{l}$ in 0.1 mM EDTA and stored at -70°C .

2.16.2. Prehybridization and hybridization

Prehybridization and hybridization conditions were modified from Maniatis (1982) and Anderson (1985); all manipulations involving nitrocellulose filters were done with gloves worn.

1. Nitrocellulose filters were wet by floating on 1% Triton X-100 solution and then immersed until evenly wet. They were then gently blotted onto 3 MM paper (Whatman) to remove excess liquid and placed inside 250 ml polyethylene bottles in which all of the following steps were performed.

2. 25 ml per 100 cm^2 of filter of NCPHY (prewarmed to 42°C) were added to the bottle which was subsequently fastened to a bottle roller in an incubator and rolled slowly at 42°C until prehybridization was complete.

3. Prehybridization solution was decanted and 10 ml per 100 cm^2 of filter of NCHY (prewarmed to 42°C) were added. Denatured probe DNA at 50 ng/ml NCHY were added to the bottle which was treated in the same way as for prehybridization, until hybridization was complete. Hybridization solution was then removed and the filters washed to the desired stringency.

2.16.3. Detection of hybrids

Hybrids were detected by binding avidin-alkaline phosphatase complexes to the hybridized biotinylated probe DNA and subsequently locating them via the precipitated formazans produced by the enzyme from BCIP and NBT.

1. All binding sites remaining on the filters were blocked by rolling the filter in 5 ml of 3% BSA dissolved in Bresa Buffer 1 for 20 min at 42 °C, followed by the addition of 5 ml Bresa Buffer 1 containing 1 µg/ml avidin-alkaline phosphatase complex. Binding was for 15 min at room temperature. The filter was then washed four times for 30 min at room temperature with 100 ml each of Bresa Buffer 1, three times for 15 min at room temperature with 100 ml each of Bresa Buffer 2 and once for 2 min with 25 ml of Bresa Buffer 3.

2. 5 ml of substrate solution containing 0.3 mg/ml nitro blue tetrazolium (NBT) and 0.2 mg/ml 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in Bresa Buffer 3 were added and the filters rolled at room temperature in subdued light until the hybrids could be visualized. The reaction was then stopped by washing the filter twice with Bresa Buffer 4, followed by drying the filter, which was stored dry between two sheets of 3 MM paper in the dark at ambient temperature.

2.17. Analysis by Northern hybridization

Homologies between DNA sequences and RNA transcripts were analyzed by Northern hybridization (Maniatis 1982, Williams 1985 and Casey 1977).

2.17.1. Transfer of RNA to nylon membranes

RNA molecules were separated under denaturing conditions as described in 2.11.3. When electrophoresis was complete, the gel was soaked 10 min in ice-cold 1 x TAE buffer and prepared for electrophoretic transfer: Unused areas were trimmed away with a razor blade and the gel was placed on a lucite plate. The sandwich was built up in the following manner: Bottommost was a piece of 0.5 inch "egg crate" louvel cut to fit snugly into the electroblotting apparatus, followed by three sheets of 3 MM paper soaked in 1 x TAE. After this the gel was placed on the filter paper with the original upper side facing down. A prewet (wetted in water and soaked in 6 x SSC until transfer) sheet of Nytran (0.22 µm pore size) was layered onto the gel, taking care not to trap any bubbles, after which it was trimmed to the exact dimensions of the gel. This was followed by three layers of soaked 3 MM paper. The sandwich was completed by a second piece of louvel of the same dimensions and secured with two rubber bands.

The sandwich was placed (in the correct polarity) into the blotting apparatus partially filled with ice-cold 1 x TAE, the buffer level adjusted to submerge it completely after which the whole apparatus was placed into an ice-water bath. Transfer conditions were: 30 min at 10 V followed by 3 hrs at 40 V, with a complete buffer change after 1.5 hrs. The sandwich was then removed and opened, the membrane peeled off the gel and washed in 6 x SSC after which it was baked in a preheated vacuum oven at 80 °C for 2 h between two sheets of 3 MM paper.

2.17.2. Prehybridization and hybridization

Prehybridization and hybridization conditions were the same as for DNA - DNA hybrids (see: 2.16.2.), with the following exceptions: Nytran membranes were not prewet before they were placed into polyethylene bottles. The ratios of solution volumes

to filter area were the same, but NYPHY and NYHY were used.

2.17.3. Detection of hybrids

Conditions for hybrid detections were the same as for DNA – DNA hybrids with the following exception: The blocking procedure was done with 5% BSA for 1 h at 65 °C.

2.18. Assay of β – galactosidase

Assays of β -galactosidase were performed with *E. coli* and *S. cerevisiae*. For *E. coli* they were modified from Miller (1972), for yeast they were modified from Rose (1983), Casadaban (1983) and Ruby (1983).

2.18.1. Assay of β – galactosidase in *E. coli*

1. Transformed *E. coli* were grown to saturation overnight in LB with antibiotic. Two parallel cultures (5 ml) of 2 x LB with antibiotic were inoculated with 100 μ l fresh overnight and incubated at 30 °C in a test tube roller. One test tube was additionally inoculated with a randomly chosen strain and used for monitoring the growth of the batch. When the OD₆₀₀ of this tube reached 0.5, all tubes were placed in an ice-water bath for 20 min after which the cells were washed once and resuspended in an equal volume of ice-cold M 10 without carbon source.

2. The OD₆₀₀ of each culture was measured, after which 0.5 ml and 0.1 ml cell suspension were added to test tubes filled with 0.5 ml and 0.9 ml Z – buffer, respectively. The latter manipulations were performed in parallel, so that every original culture was assayed in quadruplicate. Two drops of chloroform and one drop of 0.1% SDS were added to each tube, which was then vortexed immediately for 15 sec.

3. Test tubes were then placed in a water bath at 28 °C and preincubated for 5 min after which the reaction was started by the addition of 200 μ l ONPG solution (prepared as described in Miller (1972)). The reactions were stopped when sufficient color had developed by the addition of 0.5 ml 1 M Na₂CO₃ after which the samples were either measured immediately or stored on ice for maximally 1 h before measuring them. The reaction times for individual tubes were noted.

4. Optical density at λ = 420 nm and 550 nm were recorded and the units of β -galactosidase calculated with the formula given in Miller (1972).

2.18.2. Assay of β – galactosidase in *S. cerevisiae*

1. Cells to be assayed were prepared by growing freshly transformed yeast in 5 ml minimal medium after which 50 ml of the same medium in 500 ml Erlenmeyer flasks with baffles were inoculated with 1 ml and put in a rotary shaker at 30 °C and 100 rpm for 30 hrs. Cells (generally from 5 ml) were then harvested and resuspended in 2.45 ml 0.1 M KPO₄ (pH 7.0), 20% glycerol; mixed and stored at – 70 °C.

2. Frozen cells were thawed on ice and the following reactions set up: 50 μ l cells added to 950 μ l ZY buffer and 250 μ l cells added to 750 μ l ZY buffer; each of which were set up twice so that cells from one flask were assayed in quadruplicate. The re-

action mixes were vortexed for 10 sec and then incubated at 23 °C for 10 min followed by the addition of 50 μ l chloroform. The tubes were vortexed 10 sec and incubated for 10 min at 30 °C before starting the reaction by the addition of 0.2 ml ONPG (prepared as described by Miller (1972)).

3. The reactions were stopped when sufficient color had developed by the addition of 0.5 ml 1 M Na_2CO_3 after which the samples were centrifuged in a microfuge at the highest speed to eliminate interference by particulates when measuring absorbance. The supernatant was measured immediately or stored on ice for maximally 1 h before measurement.

4. Optical density at $\lambda = 420$ nm and 550 nm (as a control) were recorded and the units of β -galactosidase calculated by dividing the absorbance at 420 nm by the product of reaction duration (in minutes), volume of cell suspension added (in ml) and dry weight per ml and multiplying by 1000.

2.18.3. Statistical analysis of assay data

The data obtained from β -galactosidase was analyzed with Students' t-test (Leaver 1974) to determine whether the values obtained deviated significantly from the values of either a *lac*⁻ strain or a fully induced wild type strain.

2.19. Assay of cinnamic acid decarboxylase

Cinnamic acid decarboxylase assays were either done with *t*-cinnamic acid as substrate and termed CAD assays or with analogs and then termed CAAD assays. Residual substrate and evolved product were separated by HPLC; the exact conditions of chromatography are given in the Results section.

2.19.1. Assay with *t*-cinnamate as substrate

1. Yeast cells to be assayed (where not stated otherwise, from 10 ml) were collected in Pyrex screw-cap test tubes, washed once with 5 ml ice-cold 100 mM sodium phosphate buffer (pH 6.0) and frozen at -20 °C for at least 1 h. They were then lyophilized to complete dryness in the test tubes and stored at -20 °C until used.

2. The cells, made permeable through freeze-drying, were resuspended in 1 ml ice-cold 100 mM NaPO_4 pH 6.0, vortexed well and placed on ice for 5 min after which the reaction was started by the addition of 20 μ l of 50 mM Na cinnamate. The test tubes were screwed shut until they were gas-tight, placed in a water bath at 30 °C for 5 min to equilibrate them followed by vigorous agitation in a rotary shaker for 1 h at 30 °C.

3. The reaction was quenched by plunging the test tubes into liquid nitrogen, letting them equilibrate for 10 min, after which the screw caps were opened and the following solutions added very slowly with the tubes still immersed in LN_2 : 0.5 ml 4 N KOH, 0.2 ml 5 M NaCl, 3 ml purified *n*-hexane. The screw caps were fastened gas tight again, the test tubes withdrawn from LN_2 and after thawing the product extracted by vortexing at top speed for 5 min.

4. The emulsion was broken by 10 min centrifugation, followed by plunging the tube

into liquid nitrogen, thawing and spinning once more after which the phases were completely separated. The upper phase was withdrawn and either analyzed immediately or transferred to a second tube and stored at -20°C .

2.19.2. Assay with derivatives of *t*-cinnamic acid as substrates

1. Cells to be assayed with analogs of *t*-cinnamic acid as substrate were prepared identically as described above.
2. The cells, made permeable through freeze-drying, were resuspended in 1 ml ice-cold 100 mM NaPO_4 pH 6.0, vortexed well and placed on ice for 5 min after which the reaction was started by the addition of analog to a final concentration of 1 mM. The reaction was then performed identically as described for *t*-cinnamic acid until the extraction step. Extraction was performed twice in very subdued light with 5 ml each of either pentane or diethylether to which 2,6 di *tert*-butyl-4-methyl phenol (DBMP) had been added to a final concentration of 10 μM . After removal from LN_2 and thawing, the organic phase of the first extraction was transferred to a second tube and stored at -20°C until it was combined with the organic phase of the second extraction. The products of these reactions were exposed to as little light as possible.
3. The organic phases were transferred to 100 ml Erlenmeyer flasks and dried by adding a spatula full of Na_2SO_4 after which the solvent was sucked through a sintered disc filter into a tapered flask. The solvent was removed at reduced pressure in subdued light in a rotary evaporator with the tapered flask held at 0°C . The products were taken up in 5 ml of filtered methanol and stored at -70°C in complete darkness until analyzed by HPLC.

2.19.3. HPLC analysis of assays

Substances extracted from CAD and CAAD assays into the organic phase were analyzed by HPLC without further manipulations. Separations were performed on a reversed-phase C-18 column packed with 5 μm spherical particles and with 10 cm x 0.8 cm dimensions. The following mobile phases were used: For CAD assays (v/v): methanol : water, 95 / 5; for CAAD quantitative assays: methanol : water, 10 / 3; for recording spectra from CAAD assays: methanol : water, 10 / 7 followed by column flushing with methanol : water 10 / 1. Chromatograms and spectra were recorded at the wavelengths given in results.

2.19.4. Quantitation of HPLC data

Quantitation of HPLC data was achieved by integrating peak areas from peaks separated by chromatography and calculating the amount of product present in the sample with a linear equation derived from the peak areas of standards with known concentrations. For the quantitation of the decarboxylation products of *t*-cinnamic acid analogs which were not available commercially an indirect approach was used: A factor, calculated from the ratio of published molar extinction coefficients of decarboxylation product and a set of standards was inserted into the linear equation to determine amounts of product.

2.20. Genetic methods for *S. cerevisiae*

General genetic methods for yeast were as described by Sherman (1983).

2.20.1 Mutagenesis

1. Stationary phase yeast cells to be mutagenized were washed twice in sterile water followed by one wash in 100 mM Na acetate pH 4.5. Cells were then resuspended in sodium acetate buffer at 1×10^8 N/ml and aerated on ice. At $t = 0$ nitrosoguanidine (MNNG), dissolved in the same buffer, was added to a final concentration of 2.5 mg/ml.

2. 1 ml cell suspension was removed at intervals, added to 9 ml YEX and placed on ice. Cells were then washed twice in YEX and incubated at 30 °C in 10 ml YEX with aeration for three hours for segregation. Aliquots of dilutions were plated on MEA.

2.20.2. Determination of reversion frequencies

Reversion frequencies for *cad* mutants were determined by plating an undiluted aliquot of a freshly grown stationary phase culture in MEA + *t*-CA top agar on MEA + *t*-CA plates and a diluted aliquot on MEA plates. The ratio of resistant colonies to the titer of viable cells was defined as reversion frequency.

2.20.3. Genetic crosses for strain constructions

1. Yeast strains for crosses were first purified by streaking them twice on YEX after which they were tested phenotypically for their auxotrophic markers. Single colonies were then streaked as lines on supplemented YEX, with up to six lines per plate, and incubated 2 d at 30 °C.

2. Cells were mated by replica plating strains of opposing mating type onto a new plate with supplemented YEX in a manner that the sets of parallel lines were then arranged perpendicularly to each other. After 2 d at 30 °C the crosses were replica plated onto plates selective for diploids.

3. Purified single colonies were then grown in 10 ml supplemented pre-sporulation medium for 2 d at 30 °C, pelleted and transferred to the same volume of supplemented sporulation medium and grown for one week.

4. Cells and ascospores were pelleted, washed twice with water, resuspended in 5 ml of 25 mM KPO_4 pH 7.5, 0.2 mg / ml Zymolyase 5 K after which 1 ml of glass beads (SIGMA type I) were added and the tube incubated for 3 hrs in a rotary shaker at 30 °C. Tween 80 was added to 2% and each tube vortexed for 5 min. Dilutions were made in 1% Tween 80, aliquots plated on YEX and incubated for 3 d.

5. Phenotypes were scored after picking single colonies to rich medium and replica plating on selective plates. Mating type was scored by making replicas onto a plate where previously a strain with known sex had been spread and selecting for diploids.

2.20.4. Generation and selection of petites

Strains to be made ρ^- were grown to saturation overnight in YG at 30 °C. 1 ml was withdrawn, washed twice with YM and resuspended in 1 ml YM. 5 ml YM supplemented with 0.01% casamino acid and 10 $\mu\text{g/ml}$ final concentration ethidium bromide were inoculated with 50 μl washed cell suspension and incubated for 5 hrs at 30 °C. Aliquots were then plated onto YGG and incubated for 4 d. Small colonies were picked and purified once on YGG.

2.21. Genetic methods for *E. coli*

General genetic methods for *E. coli* were performed as described by Miller (1972). *ThyA* mutants were selected by plating fresh overnight cultures on NBTT, incubating at 30 °C for up to 5 d, purifying the small colonies that arose by restreaking on the same plates. *RecA* strains were constructed by transferring this marker by conjugation as described by Miller (1972) and Schleif (1981) and selecting against the parents.

3. Results

3.1. Biochemistry of *t*-cinnamic acid decarboxylase

3.1.1. *t*-cinnamic acid inhibits growth of *CAD* yeast

The growth of wild type yeast was studied under different conditions in order to reveal the nature of growth inhibition due to *t*-cinnamic acid. It is well known for lipophilic acids like *t*-cinnamic acid that the uncharged form can freely permeate the cell membrane, the ionic form, however, cannot (De La Peña 1982). This results in an equal internal and external concentration of the uncharged species, whereas the intracellular concentration of the ionic form is generally higher than the external one due to the relative alkalinity of intracellular pH caused by electrochemical proton export.

Growth of GRF 18 at pH 5.6

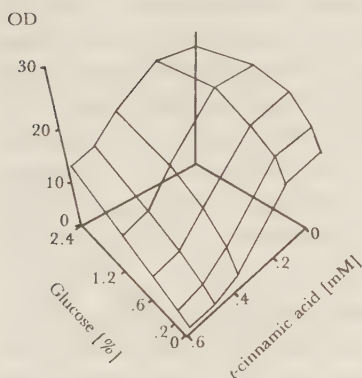


Figure 1 OD₆₀₀ plotted as a function of *t*-cinnamic acid and glucose concentration. Cells were grown for 17 hrs at 30 °C with constant agitation in 5 ml MEA buffered with 100 mM sodium citrate at pH 5.6. The test tubes were inoculated with 10⁴ cells each from a fresh stationary culture. The pH was measured before and after growth. It did not change by more than .2 pH units. Absorbances were measured from diluted samples. Each node in the graph represents the mean of two measurements.

Growth of GRF 18 at pH 6.5

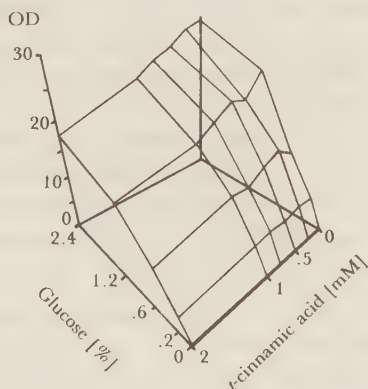


Figure 2 OD₆₀₀ plotted as a function of *t*-cinnamic acid and glucose concentration. Cells were grown for 17 hrs at 30 °C with constant agitation in 5 ml unbuffered YEX. The test tubes were inoculated with 10⁴ cells each from a fresh stationary culture. The pH was measured before and after growth, it did not change by more than .2 pH units. Absorbances were measured from diluted samples. Each node in the graph represents the mean of two measurements.

The growth inhibitory effect of *t*-cinnamic acid on wild type yeast was expected to be pH dependent, similar to the effects shown by other lipophilic acids such as benzoic acid. Figures 1 and 2 show the final OD₆₀₀ of wild type yeast grown at two different pH values, plotted as a function of glucose and *t*-cinnamic acid concentration. Growth inhibition by *t*-cinnamic acid was a function of pH, with inhibition increasing as the pH decreased. Glucose alleviated this growth inhibition.

The next set of experiments was designed to answer the question whether growth inhibition was caused by influencing transport across the cell membrane. Carruthers *et.al.* (1986) have demonstrated that the addition of lipophilic solutes to synthetic bilayers caused changes in the molecular order of the lipid membrane. These changes resulted in pronounced non-linear effects on the catalytic properties of a membrane protein. In order to discriminate between effects on transport and possible effects on cellular energy metabolism (Krebs 1983), an additional inhibitory substance, *p*-*fluoro* phenylalanine, was included in the growth medium. The growth medium contained 100 mM NH₄⁺. The general amino acid permease (*GAP1*) system, with which external L-phenylalanine and, presumably, also *p*-*fluoro* phenylalanine is taken up into cells (Cooper 1982) is repressed under these conditions (Roon 1975 and Cooper 1982). If *t*-cinnamic acid promotes the influx of *p*-*fluoro* phenylalanine, the two inhibitory substances together are expected to show synergistic effects in growth inhibition of *S. cerevisiae*. The results of these experiments are shown in figures 3, 4, 5 and 6. Both substances together exerted a pronounced, synergistic growth inhibitory effect on *S. cerevisiae* GRF 18.

An experiment to measure changes in intracellular pH as well as permeation enhancement due to treatment of yeast cells with *t*-cinnamic acid was performed. Stationary phase *CAD* yeast cells were suspended in buffer and placed into the microwave cavity of an epr spectrometer. The buffer contained free nitroxide radicals which partition between the intra- and extra-cellular space analogous to lipophilic acids and equilibrate in the intracellular space into ionic and neutral species as a function of pH. The external signal was quenched by the addition of ferricyanide. The objective was to measure the Δ pH and the kinetics of this change after addition of *t*-cinnamic acid (Cafiso 1981). However, no intracellular signal could be recorded.

Figure 3

Growth of GRF 18 at pH 5.5
with no *p*-fluoro phenylalanine

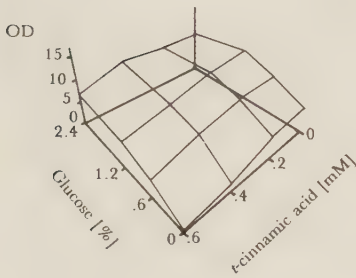


Figure 4

Growth of GRF 18 at pH 5.5
with 125 μ g/ml *p*-fluoro phenylalanine

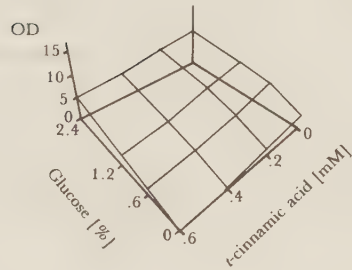


Figure 5

Growth of GRF 18 at pH 5.5
with 250 μ g/ml *p*-fluoro phenylalanine

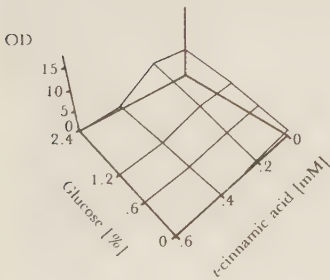
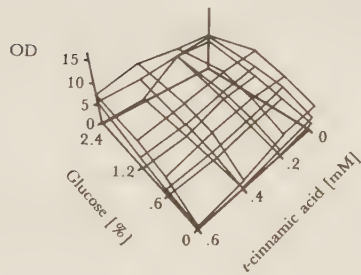


Figure 6

Growth of GRF 18 at pH 5.5



Figures 3, 4, 5, and 6 OD₆₀₀ plotted as a function of *t*-cinnamic acid and glucose concentration. Figure 6 is a composite from the preceding three figures. Cells were grown for 17 hrs at 30 °C with constant agitation in 5 ml MEA buffered at pH 5.5 with 100 mM NH₄ citrate. *p*-fluoro phenylalanine was added to the medium at two different concentrations. The tubes were inoculated with 10⁴ cells each from a fresh stationary culture. The pH was measured before and after growth, it did not change by more than 0.2 pH units. Samples were diluted to absorbancies under 0.5. Each node in the graph represents the mean of two measurements.

3.1.2. *S. cerevisiae* decarboxylates *t*-cinnamic acid and cinnamic acid analogs to styrene and substituted styrenes

Decarboxylation of *t*-cinnamic acid

Previous observations that *S. cerevisiae* possesses an enzyme responsible for the decarboxylation of *t*-cinnamic acid to styrene (Chen 1957, Albagnac 1975, Goodey 1982) were confirmed in this work. Yeast growing on media containing *t*-cinnamic acid gave off the pungent odor typical for styrene. Enzyme reactions were performed with *t*-cinnamic acid as substrate. After extraction into an organic phase the products were analyzed by HPLC. A chromatogram of these products (figure 7), compared with a chromatogram of standard (figure 8) does not reveal any differences. The retention times of the biogenic and synthetic styrene were indistinguishable.

Figure 7

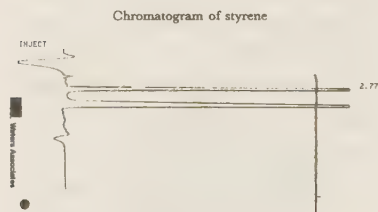
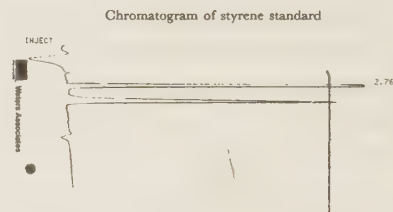


Figure 8



Figures 7 and 8 illustrate chromatograms of biogenic and synthetic styrene (2.77 min and 2.76 min), respectively. Assays and HPLC analysis were performed as described in Methods. The left trace is a plot of absorbance at 246 nm; the right trace is a plot of system pressure. The small peak preceding styrene is the void volume peak, the large peak following it is from the solvent, *n*-hexane.

The pH optimum of the enzymatic reaction was determined by performing reactions in six different buffers. The results from these experiments are plotted in figure 9. The pH optimum of the enzymatic reaction performed with lyophilized cells was at pH 6. A broad asymmetric peak of optimal activity existed with activity rapidly declining below pH 6. At the same pH, activity was higher when assays were done in non-chelating buffers such as MES and TES than assays performed in citrate buffer. Phosphate buffer was used in all further assays; they were termed standard assays.

Figure 9

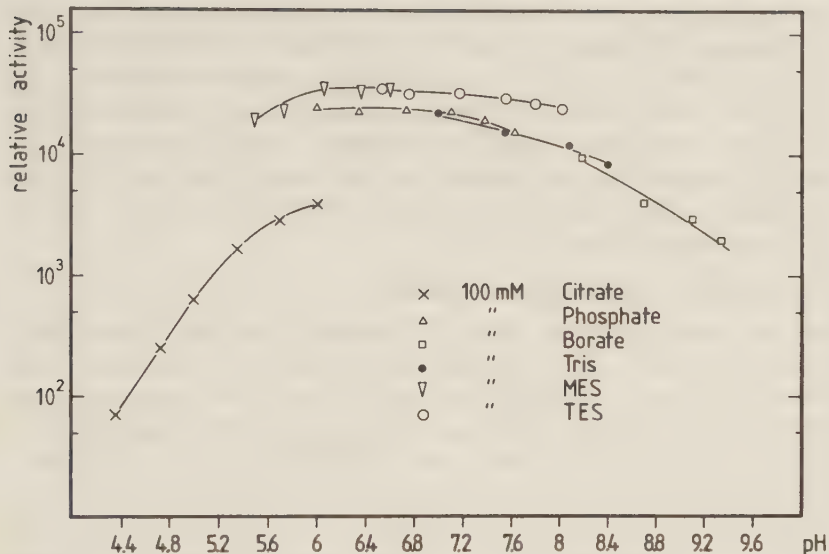


Figure 9 is a plot of specific *t*-cinnamic acid decarboxylase activity as a function of assay buffer and pH. Specific activity is expressed in arbitrary, dimensionless units, termed relative activity and plotted on a logarithmic scale. Each point on the graph is the mean of the results obtained from two determinations.

Figure 10

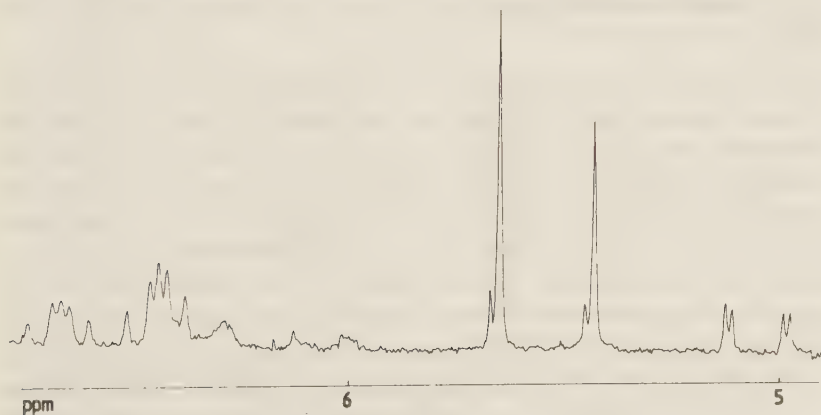


Figure 10 shows the ^1H 80 MHz NMR spectrum of the vinyl proton region of styrene. A standard reaction was performed in D_2O , pH was adjusted to 6 with NaOD. The products were extracted into toluene- d_8 as described in Methods.

The stereochemical course of the reaction was investigated to obtain more information on the mechanism of decarboxylation. The reaction was set up as described in the legend of figure 10 and the product extracted was analyzed by NMR. Figure 10 shows the ^1H NMR spectrum of styrene in the vinyl group region. The spin coupling constants and the chemical shifts of the protons in this region allowed the position of the ^2H atom to be assigned to the one previously occupied by the carboxyl function. The original geometry about the double bond was retained. Approximately 80% of the molecules were labeled with ^2H .

Decarboxylation of *t*-cinnamic acid analogs

Previous observations that analogs of *t*-cinnamic acid are decarboxylated with different efficiencies by yeast (Albagnac 1975, Gramatica 1981, Tubb 1980, Wackerbauer 1978) were confirmed in this work. Assays were performed with the following substances as substrates: 2-OH cinnamic acid, 4-OH cinnamic acid (*p*-coumaric acid), 3,4 *di*OH cinnamic acid (caffeic acid), chlorogenic acid (the quinic acid ester of caffeic acid), 3-OCH₃, 4 OH cinnamic acid (ferulic acid), 3-NO₂ cinnamic acid and 4-NO₂ cinnamic acid. All substrates gave rise to decarboxylated species. UV spectra of the reaction products, separated in advance by HPLC are shown in figures 11 to 16. The amounts of decarboxylation products obtained in the assays with caffeic acid and chlorogenic acid were too small to be quantified accurately. The spectrum designated as 3,4 *di*OH styrene (figure 13), was obtained from the product of the assay with chlorogenic acid. The λ_{max} values observed were identical to those reported in the literature (see table 1 for sources).

Quantitation of the reaction products was performed as follows: A set of equations was developed from the peak area data of the standards used for calibration (styrene, *m*-cresol and 3-*nitro* styrene, figure 17). Amounts were then calculated by multiplying the results with the relative extinction factors listed in Table 1. This was done independently for all three standards (other relative extinction factors not shown). The average values from these calculations are shown in figure 18. The highest decarboxylating activity with naturally occurring substances was observed when ferulic acid was used as substrate. The activity with *t*-cinnamic acid and *p*-coumaric acid as substrates were approximately equal. However, if both of these were assayed simultaneously, *p*-coumaric acid was preferred over *t*-cinnamic acid as substrate. The highest overall specific activity was observed for the synthetic substrate 4-NO₂ cinnamic acid.

Figure 11

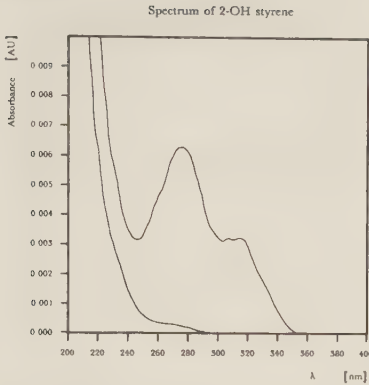


Figure 12

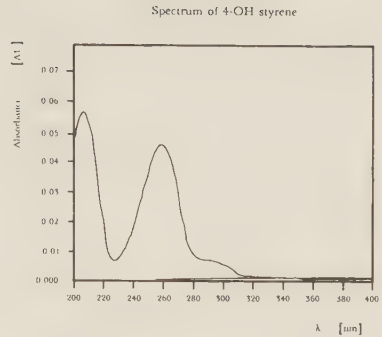


Figure 13

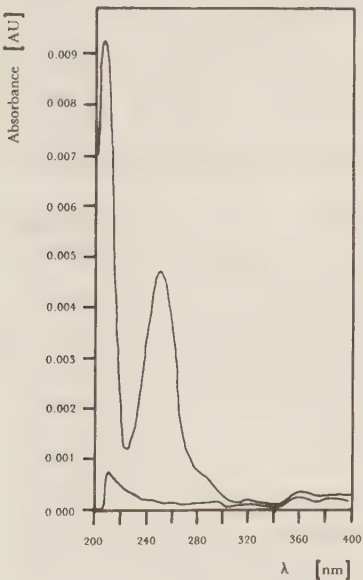
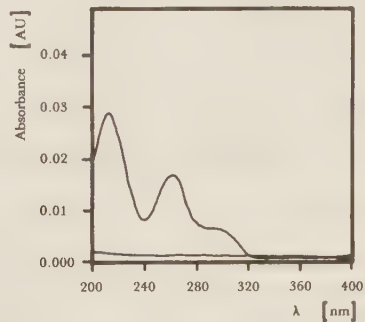
Spectrum of 3,4-*di*-OH styrene

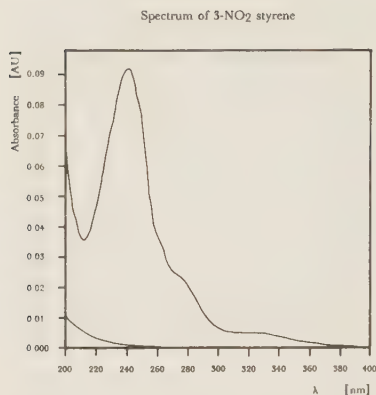
Figure 14

Spectrum of 4-OH, 3-OCH₃ styrene

Figures 11, 12, 13, and 14 show UV spectra of decarboxylation products of naturally occurring phenylacrylic acids (top trace in each figure). Products were separated by HPLC, flow was then stopped when the compound sought was in the detection cell, which was then scanned for the indicated interval. Background was subtracted by performing the same sequence with no sample injected,

flow being stopped at the same time the sample would elute. The values obtained from the scan of the background were stored and subtracted automatically from the absorbance values of the samples. The lower trace in the figures shows a scan of the background performed immediately after the measurement of the substituted styrene.

Figure 15



Figures 15 and 16 show UV spectra obtained from synthetic analogs as described in the preceding caption.

Figure 16

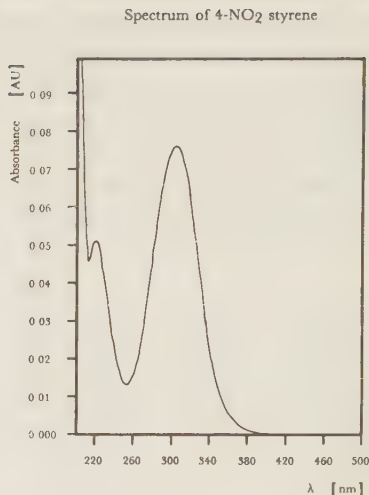


Table 1

Molar extinction coefficients of the products of CAAD assays

Substance	ϵ at measured wavelength	extinction relative to styrene
Styrene	15136 (1)	100.0
2-OH styrene	10965 (2)	72.4
4-OH styrene	16982 (3)	112.2
3-NO ₂ styrene.	23988 (4)	158.5
4-NO ₂ styrene	13804 (5)	91.2
4-OH,3-OCH ₃ styrene	— (6)	162.7
<i>m</i> -Cresol	1549 (1)	10.2

(1) (Stadler Collection)

(2) (Oki 1966)

(3) (Finkle 1962)

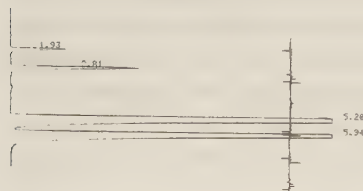
(4) (Joy 1959)

(5) (Kamlet 1955)

(6) The extinction factor for 4-OH,3-OCH₃ styrene was estimated by multiplying the hyperchromicity factor from 2-OCH₃,*p*-cresol versus *p*-cresol (see 1) with the extinction coefficient of 4-OH styrene. A further estimate of this extinction factor was also made from a spectrum of 4-OH,3-OCH₃ styrene kindly provided by V. Andreoni (personal communication). This resulted in an extinction factor of 150.6 relative to styrene.

Figure 17

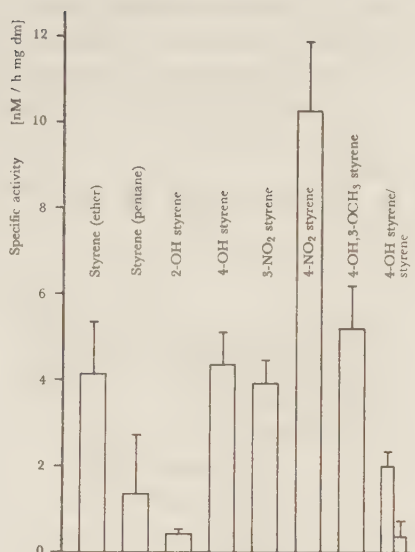
Chromatogram of standards for CAAD assay



Figures 17 and 18: Fig 17 shows a chromatogram of the standard used in the quantitation of CAAD assays separated by HPLC. *m*-cresol eluted at 2.81 min; 3-NO₂ styrene at 5.20 min and styrene at 5.94 min. Fig 18 shows the specific activity of the enzyme plotted as a function of the substrate species. Substrate concentration was 1 mM. Evolved product was quantified as described in the text with the factors from table 1. Both substrates were present simultaneously (1 mM) in the assay quantified in the last column. Assays were performed at pH 6 in phosphate buffer.

Figure 18

Quantitation of products from CAAD assays



Inhibitors and activators of the enzymatic reaction

The search for inhibitors and activators of enzymatic activity was expected to reveal more information on the nature of the enzymatic reaction. The lower specific activities in assays performed in citrate buffer suggested the necessity of a cationic cofactor for the enzymatic reaction. Standard assays were performed with the addition of either EGTA or CDTA as complexants. Quantitative analysis of these reactions revealed an insignificant reduction in activity due to EGTA but a distinct reduction when CDTA was added (figure 19). Assays were performed subsequently with CDTA and with various bivalent cations added. The addition of Zn²⁺ and Cd²⁺ resulted in a partial, the addition of Mg²⁺, in an almost complete restoration of the original activity (figure 19). The addition of either Co²⁺ or Mn²⁺ to the assay resulted in an enzymatic activity enhanced over the initial, uninhibited one.

A large proportion of decarboxylating enzymes known, require cofactors (Mahler 1971, Recsei 1984). Most amino acid decarboxylases require

pyridoxal phosphate, pyruvate decarboxylase requires thiamine pyrophosphate whereas a few enzymes, e.g. histidine decarboxylase of *Lactobacillus* 30a (Recsei 1984) require pyruvate as cofactor. These cofactors participate in very different enzymatic reaction mechanisms but are generally involved in charge stabilization. Their possible involvement in the decarboxylation of *t*-cinnamic acid was examined. The amounts of styrene that evolved in standard assays performed with the addition of each of these substances (at 1 mM/l) did not deviate significantly from the control reaction (results not shown).

Figure 19

relative activity

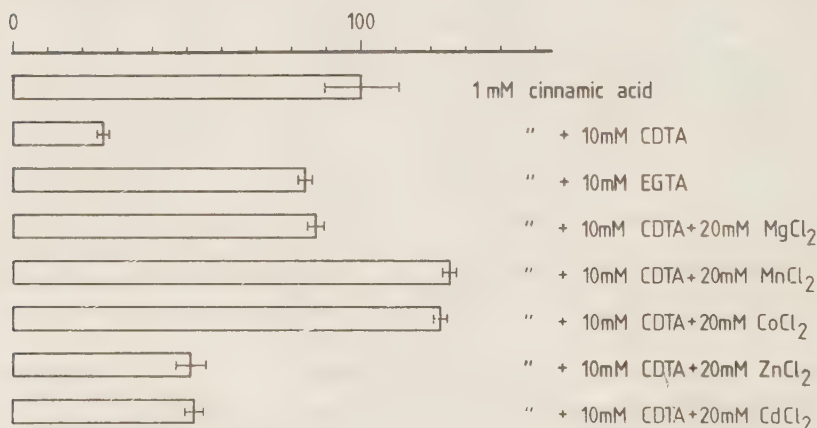


Figure 19 illustrates the effect of the addition of complexants to the assay on the specific activity of *t*-cinnamic acid decarboxylase. The specific activity of the reaction was normalized to arbitrary, dimensionless units, with the control set to 100 units. In the reactions with complexant and cation added, the cation was added after 10 min equilibration time on ice. Substrate was added last.

A possible involvement of *t*-cinnamic acid decarboxylase in the tricarboxylic acid cycle was examined. However, the addition of oxalacetate, *t*-aconitate, 2-oxoglutarate or succinate to standard assays did not show any significant effect on the amount of styrene produced (results not shown).

3-(4-imidazolyl)-acrylic acid (urocanic acid) and 3-(3-indolyl)-acrylic acid are intermediates in the degradation of L-histidine and L-tryptophan, respectively. In humans, the presence of 3-(3-indolyl)-acrylic acid in urine is a pathological condition; however, liver and kidney slices of rats convert indolylpropionic acid to the corresponding acrylic acid (Smith 1968). It is

not known whether the two compounds mentioned above are intermediates in the degradation of L-histidine and L-tryptophan in *S. cerevisiae* (Cooper 1982). A standard assay was performed with the addition of each substance. The presence of urocanic acid in the assay did not result in any significant change in the amount of styrene produced (not shown); whereas in the presence of 3-(3-indolyl)-acrylic acid (1 mM/l) the amount of styrene produced in the assay was reduced to 35% of the control (figure 20).

Figure 20

Effect of analogs on the enzyme activity of *t*-cinnamic acid decarboxylase

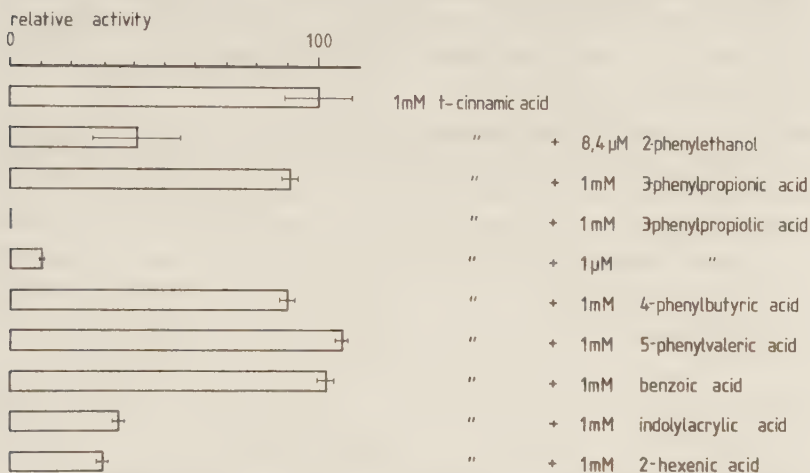


Figure 20 shows the effect of the addition of various analogs to the assay on the specific activity of *t*-cinnamic acid decarboxylase. The specific activity of the reaction was normalized to arbitrary, dimensionless units, with the control set to 100 units.

The influence of the presence of structural analogs of *t*-cinnamic acid in the reaction mixture on the activity of the enzyme was examined. The following analogs with a saturated or no side chain: benzoate, 3-phenylpropionate, 4-phenylbutyrate and 5-phenylvalerate were examined. None of these significantly changed the amount of styrene evolved, when added to a standard assay (figure 20). However, addition of the analogs 2-hexenic acid (figure 20) and 4-phenyl,3-butenic acid (data not shown) to the assay reduced the amount of product evolved to 30% and 74% of controls, respectively.

The influence of L-phenylalanine, 3-phenylpropionic acid (the alkynyl analog of *t*-cinnamic acid) and α -aminooxy- β -phenylpropionic acid on the enzymatic decarboxylation of *t*-cinnamic acid was studied. The latter two are

potent inhibitors of phenylalanine ammonium lyases (Amrhein 1977; Hodgins 1971). Addition of L-phenylalanine to the assay did not show any significant change in the amount of styrene produced, inclusion of 1 mM/l α -aminooxy- β -phenylpropionic acid in the assay resulted in a reduction of the amount of styrene evolved to 84% of the control (data not shown). The most potent inhibitor of the decarboxylation reaction was 3-phenylpropionic acid. Addition of this compound to the assay even at 1 μ M/l resulted in an almost complete inhibition (figure 20).

The effect of 2-phenylethanol (Bohn 1974; Meade 1983) was investigated. Growth inhibition by this substance is believed to be mediated by its effect on the cytoplasmic membrane. The inclusion of 8.4 μ M/l 2-phenylethanol in the reaction mixture reduced the amount of styrene evolved to 41% of the control reaction (figure 20).

None of the substances described in this section could serve as a substrate. In no case was an additional peak detected in the HPLC chromatogram.

3.1.3. CAD activities in other organisms

Hydroxycinnamic acids such as *p*-coumaric acid and their carboxymethyl derivatives such as ferulic acid are probably universally present in higher plants (Goodwin 1983). It is very improbable then, that *S. cerevisiae* should be the only organism to have evolved an ability to convert a class of growth inhibitory substances into highly volatile products. The capability for this reaction has been demonstrated for a great number of species: *Aspergillus niger* and *Penicillium glaucum* (Herzog 1908), a large number of *Enterobacteriaceae* (Lindsay 1975), *Bacillus pumilis* (Indahl 1968), *Aerobacter* spp (Finkle 1962) and *Pseudomonas cepacia* (Andreoni 1984). In this work, it was investigated whether *Fusarium oxysporum*, *Escherichia coli* and *Schizosaccharomyces pombe* show CAD activities.

It was demonstrated previously that *Fusarium oxysporum* possesses an inducible, *t*-cinnamic acid decarboxylating activity (R. Megnet, unpublished results). The product of this reaction is styrene. These results could be confirmed in this work. It was further demonstrated, that 3-nitro cinnamic acid can serve as substrate, however, this acid was unable to induce the expression of the gene (P. Doerner, A. Ross; unpublished results).

There was no indication of an enzymatic activity on *t*-cinnamic acid with styrene as product in *Escherichia coli* (results not shown).

Schizosaccharomyces pombe cells growing on MEA plates with (1.0 mM/l) *t*-cinnamic acid were partially inhibited. At this concentration, *S. cerevisiae* had ceased to grow. However, the pungent odor typical for styrene was not detected. A standard assay performed with lyophilized *S. pombe* cells confirmed the absence of CAD activity (figure 21). Under the specific HPLC conditions used, the styrene peak was expected to appear in the chromatogram with a retention time of 2.93 minutes.

Figure 21

Chromatogram of assay from *S. pombe*

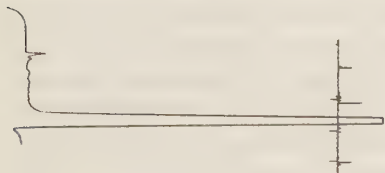


Figure 21 is a reproduction of an HPLC chromatogram. The organic phase of an assay with lyophilized *S. pombe* cells was analyzed as described in the Methods section. The trace proceeds from top to bottom; the top peak is the void volume peak, the bottom one is from the solvent, *n*-hexane. Integration was inactivated except for the duration of a time window where styrene was expected.

3.2. Genetics of *t*-cinnamic acid decarboxylase

3.2.1. Isolation of cad^- mutants

Mutants with reduced enzyme activity were a precondition to obtain more information on the genetic nature of the locus. First of all, the existence of unconditional mutants sensitive towards *t*-cinnamic acid, designated as cad^- mutants, would imply that the absence of this gene product is not lethal for *S. cerevisiae*. Furthermore, if a strategy of cloning by complementation was to be adopted, these mutants were a prerequisite for cloning the gene. MNNG (1-methyl-3-nitro-1-nitrosoguanidine) was chosen as a mutagen because of its high frequency of mutation induction and concomitant low rate of killing (Sherman 1983). MNNG primarily appears to attack GC base pairs and modify guanine by alkylation, although not all GC pairs are equally susceptible to this agent (Prakesh 1973). In yeast, this premutational lesion is fixed into a mutation predominantly by error prone repair (Lawrence 1982), resulting specifically in a transition to an AT base pair.

Figure 22

Viable cells after MNNG mutagenesis

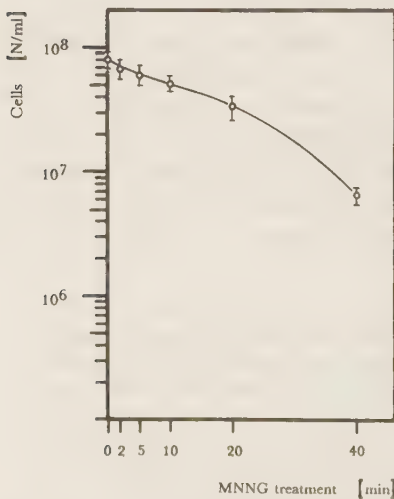


Figure 22 is a plot of the number of viable cells as a function of duration of treatment with mutagen. Aliquots were plated in triplicate.

Stationary phase yeast cells of strain GRF 18 were mutagenized as described in Methods. After the number of viable cells remaining (figure 22) was determined, an aliquot of the cells which had been mutagenized for 10 minutes (67% survival) were plated on MEA. Approximately 25 thousand colonies were obtained and further analysed. Approximately 50 clones were ade^- mutants, as judged by their red color. Mutagenesis was therefore assumed to have been sufficient to reasonably expect the generation of cad^- mutants. After growth on this non-selective medium, the colonies were replica plated onto YM supplemented with L-histidine and L-leucine and onto MEA with 0.5 mM *t*-cinnamic acid added. Selection was based on the assumption, that mutants with reduced enzyme

activity would be more sensitive towards *t*-cinnamic acid than the wild type. After four days, growth was scored. Putative mutants were required to grow on the minimal medium but not on the **MEA** plates with 0.5 mM *t*-cinnamic acid added. 172 clones fulfilled these conditions. They were picked, purified by streaking on **MEA** and retested, after which 27 were discarded because they failed to perform as required.

I had previously observed, that spontaneous *petites* generated from the cad^+ strain GRF 18 lost their resistance to *t*-cinnamic acid concomitantly with their respiratory proficiency. In order to discriminate between cad^- mutants and clones phenotypically cad^- due to a pleiotropic effect of the *petite* mutation, the respiratory status of the remaining clones had to be evaluated. The results were expected to be important in two aspects. First, the existence of respiratory proficient cad^- clones would imply a nuclear location for the *t*-cinnamic acid decarboxylase gene. Further, a strategy of gene isolation by complementation critically depends on a genetic environment which allows the expression of the gene sought. To test for respiratory proficiency, the remaining clones were replica plated onto YG, 78 of these were ρ^+ as inferred from their growth on glycerol as sole carbon source.

Figure 23

relative activity

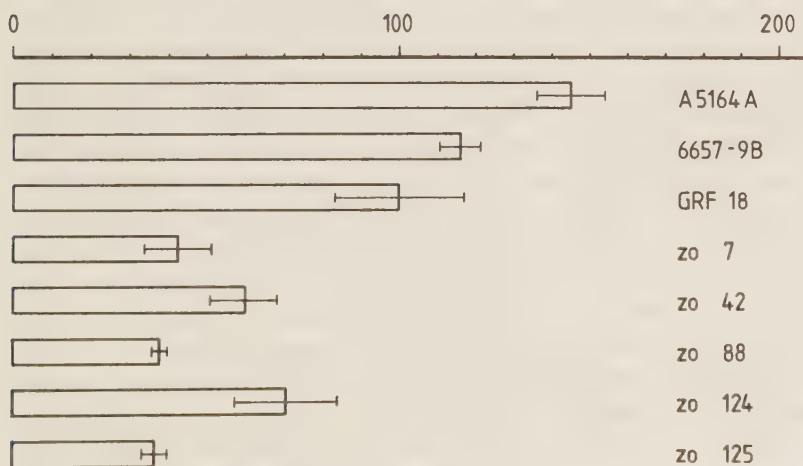


Figure 23 is a plot of residual *t*-cinnamic acid decarboxylase activity of some *z0* mutants. Specific activity is expressed in arbitrary units, normalized to the activity of the progenitor strain of the mutants, GRF 18.

The residual *t*-cinnamic acid decarboxylase activity of these 78 clones was then determined. Standard assays were performed with lyophilized cells. The amount of styrene produced was measured by HPLC after it had been extracted into *n*-hexane. Low residual enzyme activity, but growth rates not differing substantially from those of the untreated GRF 18, were the criteria by which clones were selected for further research. Eight clones were chosen and termed z0 mutants. The residual activity of some of these clones, compared to the wild type they derived from, is shown in figure 23. The residual *t*-cinnamic acid decarboxylating activity of the *cad*⁻ mutants was in the range of 35-70% of the progenitor wild-type. Strain 6657-9B, which is isogenic to GRF18 except for mating type and a different mutation in the L-histidine biosynthetic pathway, had a slightly higher decarboxylating activity than GRF18. *S. cerevisiae* strain A5164A, which was not further used in this work, showed a *t*-cinnamic acid decarboxylating activity almost 50% higher than GRF18.

The frequency of reversion to *cad*⁺ in these mutant strains was then determined. A high genetic stability of these strains was considered essential for all further investigations. Reversion frequencies were determined as described in Methods. The frequency of reversion from *cad*⁻ to *cad*⁺ was between 6.3×10^{-6} and 3×10^{-8} per cell.

3.2.2. Genetic crosses and construction of strains

Genetic crosses were performed in order to analyze the genetic nature of the *t*-cinnamic acid decarboxylase locus in greater detail. As a means to this end, crosses were also performed to introduce other genetic markers and to obtain strains with the opposite mating type. The experiments described in this section were designed to answer the following questions: Do all the isolated mutants belong to one complementation group? Assuming a single complementation group, is the wild type allele dominant over the mutant alleles? Finally, is *t*-cinnamic acid decarboxylase encoded by a nuclear gene?

In order to address the former two questions, strains with appropriate markers first had to be constructed. The $z0$ mutants were mated with the *ura 3* *ws* strain and sporulated as described in Methods. A test of this *ura3* strain performed in advance, revealed wild type levels in enzyme activity (results not shown). This precluded the introduction of a further *cad*⁻ allele into the strains to be tested. The haploid progeny were tested phenotypically for their auxotrophic markers and tested genetically for their mating type. Those strains that were relevant for further experiments are listed in 2.2.3..

It was now possible to perform a complementation analysis. All strains carrying *cad*⁻ mutations of different origin were selfed as well as mated with the other mutant strains. Most crosses with each *cad*⁻ mutation were performed in duplicate or in triplicate. In these cases, they were reciprocal in respect to mating type. This was done to detect any polar effect caused by mating type. Any bias in the results due to chance events was eliminated by evaluating at least 100 colonies per cross. The diploid progeny from each cross were picked and transferred to MEA. Replicas were then made on MEA plates with 0.6 mM *t*-cinnamic acid and on YG plates. After four days the plates were scored for growth. The results from this experiment are summarized on the eight rightmost columns of table 2. All progeny of each cross showed equal properties. All progeny of each class of matings also showed equal properties, thus excluding any polar effects. The diploid progeny were all respiratory proficient (results not shown). None of the diploid progeny attained wild type growth when challenged with 0.6 mM *t*-cinnamic acid (table 2). However, in two crosses involving strains carrying *cad*⁻ mutations derived from $z0$ 88, $z0$ 124 and $z0$ 132, weak growth was observed after three further days.

No restoration of the wild type *cad*⁺ phenotype would be expected if the

mutants all belonged to one complementation group. This was exactly what was observed. I thus conclude that all *cad*⁻ mutants belong to one complementation group, which I designate *CAD1*.

The question, whether the wild type allele was dominant over the mutant alleles was addressed next. The strains with *cad*⁻ alleles 1-7 through 1-132 were mated with either the parental wild type GRF 18 or with strain 6657-9B, which is isogenic to GRF 18 except for mating type and a substitution in the gene responsible for L-histidine auxotrophy. If *cad*⁺ were dominant over *cad*⁻, one would expect resistance to *t*-cinnamic acid similar to wild type in the diploid progeny from these crosses.

Table 2

Growth of diploid progeny from crosses between *cad1* and *CAD1* strains of *S. cerevisiae* on selective plates

Strain or allele	GRF 18	6657-9B	1-7	1-42	1-49	1-88	1-107	1-124	1-125	1-132
GRF 18	nd									
6657-9B	+	+	nd							
1-7	nd	+	- -							
1-42	o	-	- -	- - -						
1-49	nd	o	- -	- -	nd					
1-88	nd	o	- -	-	-	- -				
1-107	-	o	- -	- -	-	-	- - - -			
1-124	+	nd	-	- -	-	o	-	nd		
1-125	-	+	- -	- -	-	- -	- -	-	- - -	
1-132	o	-	-	-	nd	nd	-	o	-	- -

Legend:

nd no cross
 - no growth
 o delayed growth
 + wild type level growth

(The number of symbols per line denotes the number of independent crosses)

After mating, the diploids were purified and picked onto YEX plates. These were then replica plated onto MEA plates with 0.6 mM *t*-cinnamic acid and onto YG plates. After four days the plates were scored for growth. The diploid progeny were all respiratory proficient (results not shown). The growth response of the diploids, when challenged with *t*-cinnamic acid were not uniform. Of a total of twelve matings, the progeny of only three crosses displayed wild type level resistance, whereas the progeny of five further crosses displayed growth after three further days. These results are summarized on the left hand side of table 2. The inconsistencies of the results did not allow to compellingly conclude whether cad^+ is dominant over cad^- . As preliminary hypothesis, however, dominance of cad^+ over cad^- was assumed and cad^+ was designated *CAD1*, while cad^- was designated *cad1*.

The question whether *CAD1* is a nuclear gene was addressed next. First, ρ^- mutants (*petites*) were made from strains carrying each mutated *cad1* allele as well as from the *CAD1* strain, as described in Methods. Ethidium bromide mutagenesis, performed under these conditions, predominantly yields mitochondria with large deletions in their mtDNA (ρ^- mutants). The remaining sequences are generally greatly amplified. Although clones harboring mitochondria totally devoid of mtDNA (ρ^0 mutants) are also generated under these conditions, they are phenotypically indistinguishable from ρ^- clones. No attempt was made to differentiate between these different *petites* in this work.

An experiment was then performed in which all ρ^+ *cad1* strains were mated with ρ^- *CAD1* strains, with the reciprocal crosses also performed: ρ^- *cad1* strains mated with ρ^+ *CAD1* strains. Respiratory deficient diploids are unable to sporulate (Dujon 1981). If the *CAD1* gene were encoded mitochondrially, one would not expect to obtain any respiratory proficient haploid progeny with a *t*-cinnamic acid resistant phenotype when a ρ^- *CAD1* strain was crossed with a ρ^+ *cad1* strain. Conversely, all haploid progeny issued from crosses of ρ^- *cad1* with ρ^+ *CAD1* are expected to be respiratory proficient and *t*-cinnamic acid resistant.

The strains compiled in table 3 were crossed, the diploid progeny picked and purified on minimal plates. These purified diploids were transferred to sporulation medium. Ascospores were isolated as described in Methods and germinated on YEX plates. Haploid clones were picked onto YEX. Replicas were then made on MEA, MEA plus 0.6 mM *t*-cinnamic acid and on YG. Growth was scored after four days. The results are presented in table 3. Although only respiratory proficient diploids can undergo meiosis, and

Table 3

Growth of haploid progeny from crosses of *grandes* and *petites* of *CAD1* and *cad1* strains

Strains crossed		properties of the haploid progeny		
p ⁺ strain	p ⁻ strain	<i>t</i> -cinnamic acid resistant (%)	growth on glycerol (%)	colonies tested
z0 7	GRF 18	0	95	193
GRF 18	z0 7	0	96	207
z0 7	6657-9B	37	83	237
6657-9B	z0 7	60	82	186
z0 42	GRF 18	0	87	215
GRF 18	z0 42	6	88	159
z0 42	6657-9B	1	89	219
6657-9B	z0 42	4	98	174
z0 49	GRF 18	nd	nd	nd
GRF 18	z0 49	nd	nd	nd
z0 49	6657-9B	nd	nd	nd
6657-9B	z0 49	0	66	197
z0 88	GRF 18	nd	nd	nd
GRF 18	z0 88	0	99	220
z0 88	6657-9B	1	72	186
6657-9B	z0 88	0	65	107
z0 107	GRF 18	nd	nd	nd
GRF 18	z0 107	60	84	211
z0 107	6657-9B	4	65	209
6657-9B	z0 107	4	68	110
z0 124	GRF 18	54	88	197
GRF 18	z0 124	4	92	206
z0 124	6657-9B	nd	nd	nd
6657-9B	z0 124	nd	nd	nd
z0 125	GRF 18	2	67	166
GRF 18	z0 125	6	50	213
z0 125	6657-9B	62	66	199
6657-9B	z0 125	47	55	220
z0 132	GRF 18	nd	nd	nd
GRF 18	z0 132	68	92	198
z0 132	6657-9B	nd	nd	nd
6657-9B	z0 132	72	81	197

thus only ρ^+ haploids are issued from ascospores, it was surprising that a significant but varying proportion of the progeny was respiratory deficient at the time they were analysed. In a large number of crosses, the frequency of cad^+ progeny is very low. However, *t*-cinnamic acid resistant haploids are issued in significant amounts from some crosses involving a ρ^- *CAD1* strain and a ρ^+ *cad1* strain. Conversely, in the matings involving ρ^+ *CAD1* and ρ^- *cad1* parental strains, not all progeny are ρ^+ and cad^+ , as expected for a mitochondrially located gene.

I thus conclude that the *t*-cinnamic acid decarboxylase gene is encoded in the nucleus. The results further indicate that the particular mitochondrial species present in the cell has a distinct influence on its phenotype regarding resistance or sensitivity towards *t*-cinnamic acid.

3.3. Molecular biology of *t*-cinnamic acid decarboxylase

3.3.1. Isolation of the *CAD1* gene

A strategy of gene isolation by complementation was adopted to clone the *CAD1* gene. Briefly, this approach requires the transformation of an *S. cerevisiae* strain with multiple genetic markers with a pool of recombinant plasmids representing the entire yeast genome. The recombinant plasmid pool is constructed by ligating a mixture of randomly generated fragments of chromosomal DNA to a plasmid molecule, termed vector. The vector confers the capability of autonomous replication as well as a selectable property, eg. the complementation of an auxotrophic mutation in the recipient strain, onto the whole, newly generated molecule. Transformed cells are then recovered by plating on selective medium, on which only those cells harboring a plasmid can grow by virtue of those sequences present on the vector. A second round of selection is then performed to select for the phenotype sought. Occasionally, the first selection step can be omitted and the gene of interest can be isolated directly. The plasmids harbored by the clones displaying the desired phenotype are then isolated and transformed into *E. coli*, which is more amenable to the routine steps of molecular analysis.

The ability to adopt this approach crucially depends on two conditions: the existence of a stable, multiply mutant *S. cerevisiae* strain and a genetic background in this strain allowing the expression of the phenotype to be selected for. These prerequisites were fulfilled by the *cad1* mutants isolated in this work as well as by the yeast strains with the various *cad1* alleles introduced constructed in this work.

The yeast genomic library used in this work for isolating the *CAD1* gene was a gift of D. Botstein. The genomic library was constructed using YEp24 as vector (Botstein 1982). The vector was constructed by inserting a 1.1 kb *Hind*III fragment carrying the yeast *URA3* gene and a 2.2 kb *Eco*RI fragment carrying the replication origin of the yeast 2 μ plasmid into the *Hind*III and *Eco*RI sites of pBR322 respectively. YEp24 and recombinant plasmids constructed with it will replicate in *E. coli* and *S. cerevisiae*; with 20 – 50 copies per cell (Davison 1984a) in the former and an estimated copy number of 25 to 100 copies per cell (Chevallier 1980 and Clark-Walker 1974) in the latter organism. In *E. coli*, selection can be based on the Ap^r and Tc^r phenotypes conferred upon the host as well as by the complementation of the *pyrF* marker; in yeast, selection can be based on the complementation of the *ura3* marker of the recipient strain.

The genomic library was constructed (D. Botstein, personal communication) by inserting DNA fragments larger than 6kb, generated by partially digesting high molecular weight DNA isolated from the S288C derivative DBY 939 with *Sau*3A, into the unique *Bam*H1 site of YEpl24. Aliquots of three original master copies of the library were kindly provided as stab cultures of transformed *E. coli* cells.

The degree of representation of the *S. cerevisiae* genome in each master copy of the library was tested by isolating the yeast *LEU2* gene (results not shown). *S. cerevisiae* strain z0 42 was transformed as described in Methods. Plasmid DNA was added to the protoplast suspension at approximately 1 µg per 5×10^7 protoplasts. Transformants harboring plasmids restoring independence from L-leucine supplementation were directly selected for. The individual master copies of the library (pRB112, pRB113, pRB161) yielded 8, 9 and 5 *leu*⁺ transformants per µg DNA respectively. Six transformed clones were purified by streaking on minimal medium and subsequently grown to a density of 2×10^8 cells/ml in 50 ml YM supplemented with L-histidine. Plasmid DNA was then isolated from yeast by both procedures described in Methods and rescued into *E. coli* by transformation. The acid phenol method yielded plasmid DNA preparations contaminated with less RNA. Restriction analysis was performed with eleven transformants. Double digests with *Eco*RI and *Cla*I which, among other fragments, gave rise to a 480bp fragment when the plasmid DNA of six transformants was used as substrate. This fragment is internal to the coding sequence of the *LEU2* gene as Andreadis *et.al.* (1982) have shown. The plasmid DNA of the five other transformants lacked this fragment.

The *CAD1* gene was isolated with a two-step selection procedure. Direct selection for *cad*⁺ phenotype after transformation was not employed due to a very high background of false positives (results not shown) associated with this method. *S. cerevisiae* strain PDY 102 (*leu*⁻, *his*⁻, *cad1-42*) was transformed as described in Methods. Plasmid DNA was added to the protoplast suspension at approximately 1 µg per 5×10^7 protoplasts. The protoplast suspension was plated on minimal medium without uracil after transformation and clones with *ura*⁺ phenotype were selected for. Approximately 4000 clones were obtained, picked and transferred to YM. The transformants were then replica plated onto MEA, MEA with 0.6 mM *t*-cinnamic acid added and onto YG. After five days of growth, four clones were identified with *cad*⁺ phenotype restored. These were purified by streaking on YM plates, and then grown to a density of approximately 1×10^8 cells/ml in liquid MEA and in liquid YM. Yeast cells harboring

these plasmids did not grow on liquid YM with 2% glucose. However, when glucose was reduced to 0.2%, they grew well. Plasmid DNA from these cultures was prepared by the acid phenol procedure as described in Methods and rescued into *E. coli* DH1 by transformation as described in Methods.

Seventy five randomly chosen *E. coli* transformants were picked, grown in LB and their plasmid DNA isolated. After digesting this material with *Eco*RI, *Bam*HI and *Hind*III; the sizes of the fragments separated by gel electrophoresis were determined. The plasmids rescued into *E. coli* were grouped by the distinctive restriction fragment patterns they displayed. This classification indicated the existence of eight different plasmid species rescued from the four original yeast transformants.

Table 4

Plasmids rescued from *S.cerevisiae*

Original designation	Phenotype of <i>cad</i> ⁻ yeast with plasmid	plasmid rescued into <i>E. coli</i>
T ₁ Y-2	<i>cad</i> ⁺	pPD 14
T ₁ Y-7	<i>cad</i> ⁺	pPD 19
T ₃ Y-1	<i>cad</i> ⁻	pPD 3
T ₃ Y-10	<i>cad</i> ⁺	pPD 70
T ₃ M-2	<i>cad</i> ⁻	pPD 5
T ₃ M-4	<i>cad</i> ⁻	pPD 39
T ₃ M-5	<i>cad</i> ⁺	pPD 40
T ₄ Y-7	<i>cad</i> ⁺	pPD 48

Table 4 is a compilation of the designations and properties of plasmids rescued from yeast. The left column shows the original designations of the *E. coli* colonies obtained after transformation with plasmids isolated from yeast. The index of the first letter indicates from which of the four original *cad*⁺ transformants the material used for transformation of *E. coli* was prepared from. The second letter indicates on which liquid growth medium the culture was propagated prior to plasmid isolation. The last index is a running number. The second column shows the phenotype the particular plasmid conferred on PDY 102 after retransformation. The third column gives the final designation of the particular plasmid species after restriction analysis, under which it is referred to in this work.

PDY 102 was then retransformed with a plasmid representative for each class. Approximately 100 ura^+ clones were picked from the transformants of each plasmid species and transferred to YM plates. Replicas were then made onto MEA, MEA with 0.6 mM *t*-cinnamic acid added and onto YG. After five days, growth was evaluated. The clones transformed with five plasmid species were cad^+ , whereas the transformants of three further plasmid species were cad^- . The results are summarized in table 4.

Three plasmids, which conferred a cad^+ phenotype onto the *cad1* yeast cells harboring them, were chosen for detailed analysis. The plasmids pPD14, pPD40 and pPD48 were subjected to restriction analysis with a

Figure 24

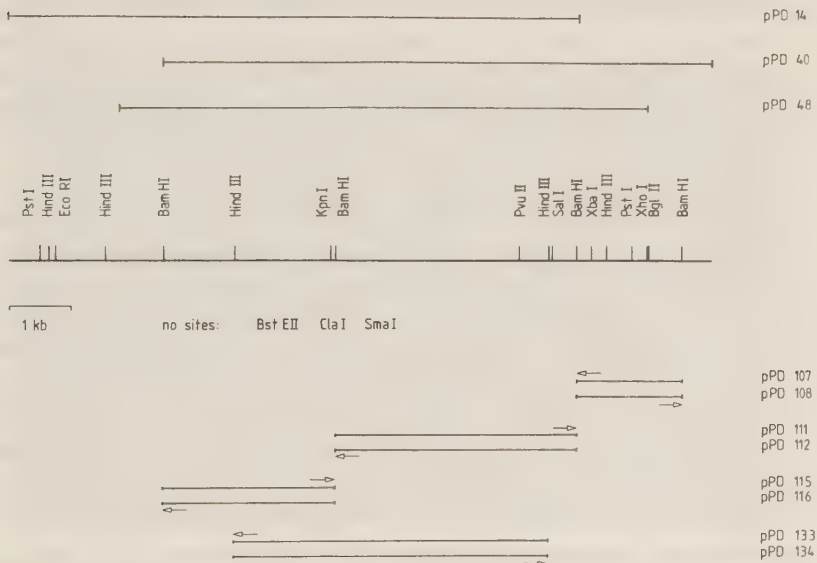


Figure 24 shows the consensus map of restriction enzyme recognition sites established from the analysis of the plasmids shown on top. The bottom part of this panel shows some of the sequences subcloned from the original plasmids and their location on the mapped stretch of yeast chromosomal DNA. The designations of the plasmids are given on the right side of the figure. The arrows indicate the orientation of the insert in the plasmid relative to the direction of transcription of the α fragment of the *lacZ* gene which continues immediately distal to the multiple cloning site in pEMBLY23.

total of 13 enzymes. A consensus restriction map of a contiguous stretch of 11.4kbp of yeast chromosomal DNA was compiled with the results from these restriction mapping experiments, which are summarized in the top half of figure 24. The plasmid pPD39, which did not confer a *cad*⁺ phenotype on transformed *cad1* yeast, was also subjected to restriction analysis. The pattern of restriction fragments obtained with the same enzymes did not reveal any similarities with the other three plasmids described above. The extent of the inserts present in these three plasmids is delineated above the restriction map in the same figure.

I then attempted to determine the location of the putative *CAD1* gene more closely. Three *Bam*HI fragments (3.95kb, 2.8kb and 1.7kb respectively) and one *Hind*III fragment (5.1kb) were subcloned into the multiple cloning site of pEMBL Y23 in both orientations (see bottom half of figure 24). This vector is part of a family of very versatile *S. cerevisiae* - *E. coli* shuttle vectors (Baldari 1985). The restriction fragments were isolated from agarose gels with Biotrap as described in Methods. They were then ligated to the vector previously cut with restriction enzyme and dephosphorylated as described in Methods. After transformation into *E. coli*, small scale plasmid preparations were made. The orientation of the inserted sequences was subsequently determined by restriction analysis.

The plasmids containing the different subcloned sequences were introduced into PDY 102 by transformation. Transformants were selected by complementation of the *ura3* marker, picked onto YM plates and replica plated onto MEA, MEA with 0.6 mM *t*-cinnamic acid added and onto YG. Growth was evaluated after five days. Figures 25 and 26 show some plates from these experiments. Clones harboring plasmids with the subcloned *Bam*HI fragments, i.e. pPD107 and pPD108 (1.7kb fragment), pPD111 and pPD112 (2.8kb fragment), pPD115 and pPD116 (3.95kb fragment) did not restore *cad*⁺ phenotype; whereas the subcloned *Hind*III fragment, pPD133 and pPD134 (5.1kb fragment) complemented the *cad1* strain and restored *cad*⁺ phenotype in either orientation.

This fragment was then characterized in more detail by restriction analysis. The plasmid DNA was digested with a further 18 restriction enzymes. The results from this restriction analysis, compiled to a restriction map, are shown in figure 27. The digestions with *Eco*RV and with *Aha*III generated more than five fragments each (results not shown). The digestion of the 5.1kb *Hind*III fragment with *Sau*3A generated at least seven fragments with the following sizes: 1.3kb, 1.1kb, 0.95kb, 605bp, 450bp, 280bp as well as two fragments with 90bp (data not shown). The positions of the

Figure 25

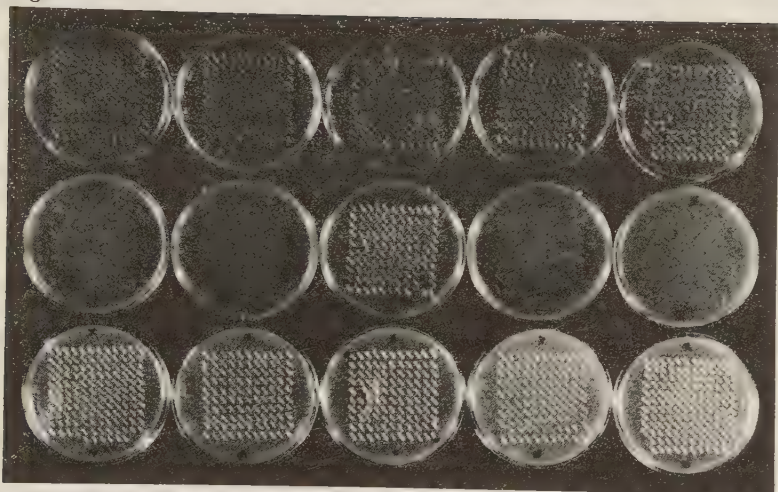
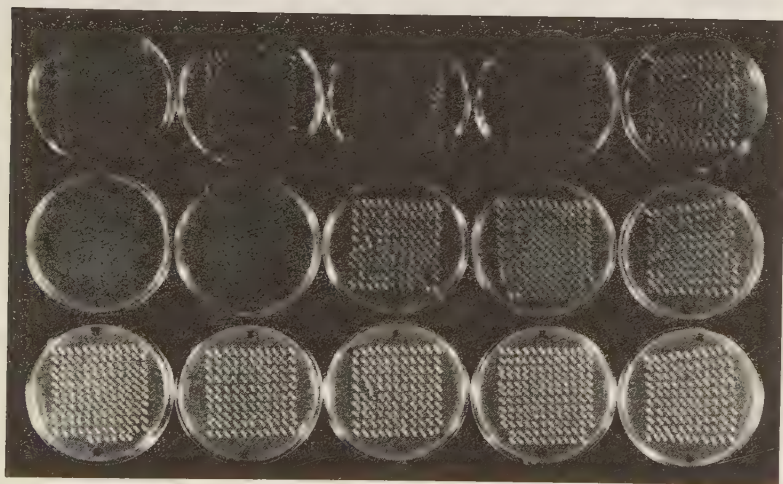


Figure 26



Figures 25 and 26 show some replica plated yeast clones picked after transformation. The plates in the top row in both panels are YG, the middle row is made up of MEA plates with 0.6mM *t*-cinnamic acid added, whereas the bottom row is made up of YM plates. The three leftmost columns in both panels are identical. From left to right; they are PDY102 transformed with YEp24 and PDY102 transformed with pEMBL23 (negative controls) as well as PDY102 transformed with pPD40 (positive control). The next column in figure 25 is PDY102 transformed with pPD111, the rightmost column in this figure is PDY102 transformed with pPD112. The next column in figure 26 is PDY102 transformed with pPD133, the rightmost column in this figure is PDY102 transformed with pPD134.

Sau3A, *EcoRV* and *AhaIII* cut sites and the relative order of these fragments were not determined.

Figure 27

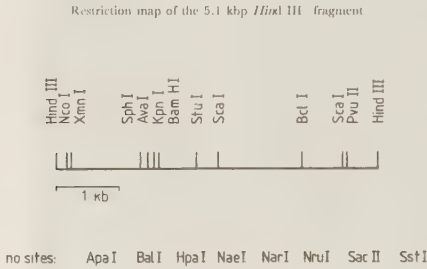


Figure 27 shows the detailed restriction map of the 5.1 kbp *HindIII* fragment which contains the putative *CAD1* gene. The map was compiled from the results obtained from digests with a total of 28 enzymes. Further to those enzymes listed in the bottom line of this figure not possessing sites on this fragment, the following enzymes do not cut in it: *BstEII*, *Clal*, *SmaI*, *PstI*, *EcoRI*, *SalI*, *XbaI*, *XhoI* and *BglII*.

The ability of the putative *CAD1* gene located on the 5.1 kb *HindIII* fragment to restore the cad^+ phenotype in strains carrying other *cad1* alleles was evaluated next. The z0 mutants do not possess a *ura3* marker, so that the pEMBLY23 vector could not be employed. The 5.1 kb *HindIII* fragment was recloned into pDB262 (Beach 1982b). This vector carries the *S. cerevisiae* *LEU2* gene and, when introduced by transformation, can restore independence from L-leucine supplementation in GRF18 and its *cad1* progeny when grown on minimal medium. This vector has the additional advantage that it can be introduced into *S. pombe* by transformation and replicate autonomously in this organism. The plasmids obtained with pDB262 as vector and the 5.1 kb *HindIII* fragment as insert in either orientation were designated as pPD150 and pPD151 respectively.

Five yeast strains carrying different *cad1* alleles were transformed together with controls. Transformed cells were selected by complementation of the *leu2* marker, picked onto YM plates and replica plated onto MEA, MEA with 0.6 mM *t*-cinnamic acid added and onto YG. Growth was evaluated after five days. In all *cad1* strains transformed with either pPD150 or pPD151, the cad^+ phenotype was restored (results not shown). After purification by streaking on YM, two single colonies from each transformation experiment were picked and grown to saturation in 50 ml of YM. The stationary phase cells were used to prepare material for an enzyme assay of *t*-cinnamic acid decarboxylase by the procedures described in Methods. When z0 mutants were transformed with vector alone, the residual enzyme activities were maximally 37% compared to the wild type progenitor strain GRF18 grown under identical conditions (see table 5). When these *cad1*

mutant strains were transformed with either pPD150 or pPD151, the specific activity measured in enzyme assays was elevated at least ten-fold over the level of the untransformed wild type GRF18 (see table 5).

Table 5

Activity of *t*-cinnamic acid decarboxylase in different yeast mutants and transformants

Yeast strain	transformed with	specific activity [nM styrene/mg dm ³ h]	activity relative to wild type (GRF 18 : 100)
GRF 18	-	3.7	100.0
PDY 102	-	0.8	21.9
PDY 102	pEMBL Y 23	0.8	21.7
PDY 102	pPD 40	26.2	707.0
PDY 102	pPD 133	29.4	795.0
PDY 102	pPD 134	26.5	715.0
z0 7	pDB 262	0.0	0.0
z0 7	pPD 150	60.0	1620.0
z0 7	pPD 151	61.0	1648.0
z0 42	pDB 262	0.0	0.0
z0 42	pPD 150	40.1	1084.0
z0 42	pPD 151	41.6	1125.0
z0 49	pDB 262	0.9	25.6
z0 49	pPD 150	43.1	1162.0
z0 49	pPD 151	61.1	1649.0
z0 107	pDB 262	0.7	19.6
z0 107	pPD 150	62.2	1680.0
z0 107	pPD 151	63.4	1710.0
z0 124	pDB 262	1.3	36.8
z0 124	pPD 150	48.9	1321.0
z0 124	pPD 151	50.1	1352.0

The size of the RNA molecule transcribed from the putative *CAD1* gene was determined next. Total RNA was prepared by the procedures described in Methods from GRF18 transformed with pPD150. The transformed cells were grown in minimal medium supplemented with uracil and adenine as well as all amino acids except L-leucine. These conditions were chosen in order to maximize the yield of the putative *CAD1* transcript. Total RNA was then separated according to size under completely

Figure 28

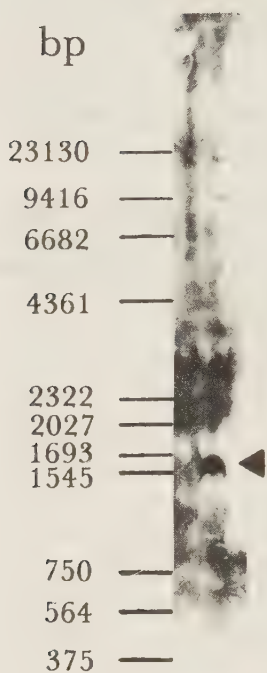


Figure 28 shows a nylon filter on which total yeast RNA separated under denaturing conditions was immobilized. The migration distances of biotinylated λ *Hind*III fragments and of biotinylated pBR322 DNA fragments cut with *Bam*HI, *Pst*I, *Pvu*II and *Eco*RI are shown on the left side. The filter was probed with single stranded pPD134. The band, marked by the arrow, was visible only when the filter was illuminated from below.

denaturing conditions, after which it was transferred to a nylon membrane (see Methods). The immobilized RNA was then hybridized to single stranded DNA recovered after superinfecting *E. coli* cells harboring either pPD133 or pPD134 with phage ϕ 1 IR1. The probe DNA had been previously photoaffinity-labelled with biotin.

In the pEMBL family of vectors, the DNA strand of the molecule which corresponds to the coding strand of the α fragment of the *lacZ* gene is encapsidated into the viral coat proteins and secreted (Dente 1983 and Baldari 1985). Depending on the orientation of the inserted restriction fragment, upon superinfection, either strand of the insert is issued as part of an otherwise identical single stranded recombinant molecule. As shown in figure 24, the insert in pPD133 is oriented in such a manner that, in the single stranded form, the recognition sequences for the following restriction enzymes present on that strand appear in the order: 5' *Hind*III *Pvu*II *Bam*HI *Kpn*I *Hind*III *Bam*HI *Eco*RI *lacZ*' 3' (the underlined sites are part of the multiple cloning site and constitute the coding strand of the α fragment of the truncated *lacZ* gene). In the single stranded form of pPD134, the order is: 5' *Hind*III *Kpn*I *Bam*HI *Pvu*II *Hind*III *Bam*HI *Eco*RI *lacZ*' 3'.

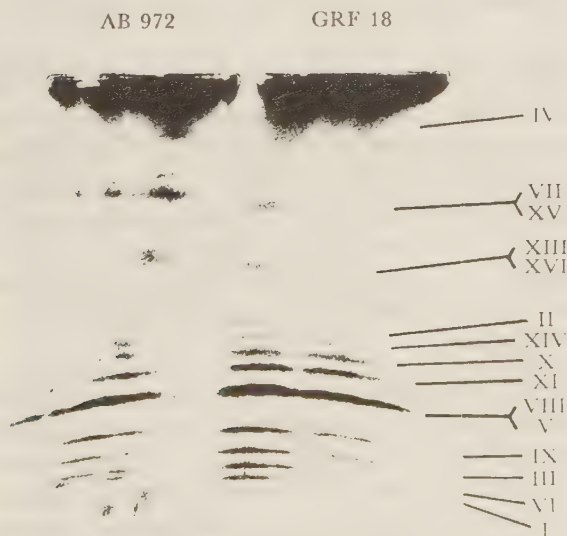
RNA-DNA hybrids not separated by the stringency washes were detected by a colorimetric reaction. A weak color reaction was seen only when single stranded

pPD134 DNA was used as probe (see figure 28). This allowed the conclusion that the coding strand for the putative *CAD1* mRNA is identical to the strand of the insert present in the single stranded form of plasmid pPD133. The coding strand is, by definition, the strand that bears the

same sequence as the messenger RNA, except, of course for possessing T instead of U (Lewin 1983). The RNA species hybridizing to the probe had migrated just further than the unresolved 1799 nucleotide long (Rubtsov 1980) cytoplasmic 18s rRNA and the 1686 nucleotide long (Sor 1980 and Li 1982) mitochondrial 16s-like rRNA species. The size of the putative *CAD1* transcript was estimated to be approximately 1600 nucleotides.

The identification of the yeast chromosome on which the *CAD1* gene is physically located was attempted next. The identification was attempted with an approach involving DNA-DNA hybridization between chromosomal DNA and previously purified and amplified sequences present on a plasmid termed probe (Southern 1975). Nitrocellulose filters with immobilized chromosomes of the yeast strains AB972 and GRF18 separated beforehand by orthogonal field alternation gel electrophoresis (OFAGE) were a gift of Ms Eva-Maria Geigl. Negatives of photographs of the OFAGE gels before transfer of the DNA to the nitrocellulose filters, stained with ethidium bromide and reproduced at original size are shown in figures 29 and 30. The individual chromosomes were identified according to Mortimer (1985) and Carle (1985) (E.M.G., personal communication). The chromosomal

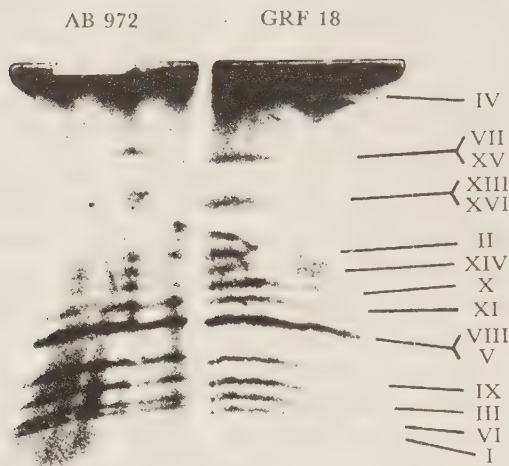
Figure 29



Figures 29 and 30 show negatives of photos of yeast chromosomes separated electrophoretically by the OFAGE method. The duration of the separation was slightly different for the gel shown in figure 30, the other parameters were identical in both cases. For visualisation, the gel was stained with ethidium bromide prior to the transfer of the DNA to nitrocellulose filters.

continued....

Figure 30



Because chromosome sizes are known in the yeast strain AB972 it was separated under the same conditions in order to facilitate identification of the chromosomal bands of strain GRF18. The bands were identified with roman numerals according to Mortimer (1985) and Carle (1985). The filters with chromosomal DNA and the photographs were a gift of Ms Eva-Maria Geigl.

DNA immobilized onto the filters was hybridized to either *Hind*III digested pPD133 (*CAD1* and *URA3* as control) or to *Hind*III digested pPD150 (*CAD1* and *LEU2* as control). The probe DNA had been biotinylated as described in Methods.

DNA-DNA hybrids resistant to the stringency washes were detected by a colorimetric reaction. Two faint bands indicating successful hybridization were detected on each nitrocellulose filter per yeast strain (shown in figure 31); on the filter of the gel shown in figure 30, the origin of migration was also visible (righthand side of figure 31). The origin was missing on the filter (lefthand side of figure 31) carrying the immobilized chromosomal DNA from the separation shown in figure 29. Thus, an unambiguous assignment of the faint bands to chromosomal bands visible on the photograph of the gel was not possible.

When the developed filter of the gel depicted in figure 30 (probed with the two *Hind*III fragments from pPD150 carrying the *LEU2* and the putative *CAD1* gene) was superimposed on the photograph of the gel, the lower, distorted band on the filter had the same distance from the migration origin as the *S. cerevisiae* chromosome II in the gel photograph. The other faint band visible on the filter revealed a migration distance indistinguishable from the electrophoretically unresolved band on the gel composed of the chromosomes VII and XV.

Figure 31

Southern blots of separated chromosomes

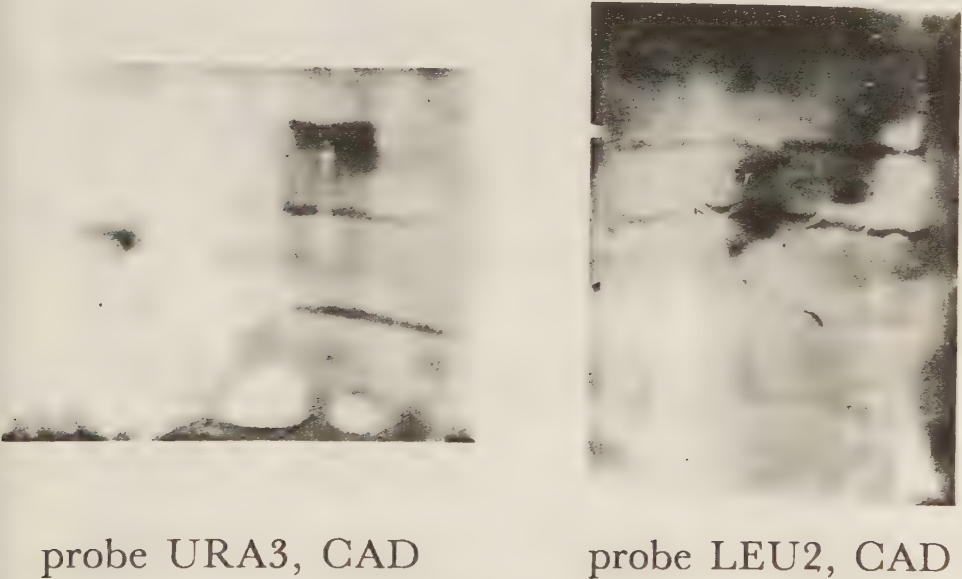


Figure 31 shows the developed filters after Southern hybridization. The nitrocellulose filter shown on the left panel was derived from the gel documented in figure 29. The filter shown on the right panel originates from the gel documented in figure 30. The filters are shown in the original size.

When the developed filter of the other gel (see figure 31) (probed with the two *Hind*III fragments from pPD133 carrying the *URA3* and the putative *CAD1* gene) was compared with the photograph of the gel (see figure 29), the best fit was obtained when the bands visible on the filter were superimposed on the bands of the photo representing the *S. cerevisiae* chromosomes II and V respectively. However, because a signal representing the migration origin was missing on the filter, this assignment remains tentative.

The *S. cerevisiae* *LEU2* gene maps to chromosome III, whereas the *URA3* gene maps to chromosome V (Mortimer 1981). The results described in the preceding paragraphs make it very probable that *CAD1* is physically located on the *S. cerevisiae* chromosome II.

3.3.2. Expression of the *CAD1* gene

The regulation of expression of the *t*-cinnamic acid decarboxylase gene was studied under various growth conditions. The experiments were designed to bear on the following questions. Does gene expression, measured as enzyme activity in the standard *in vitro* assay, depend on the carbon source in the growth medium? Is the *CAD1* gene expressed constitutively? Is the expression of *CAD1* subjected to glucose repression? Finally, considering the cad^- phenotype observed with ρ^- yeast, is *CAD1* expressed in *petites*?

S. cerevisiae GRF 18 was grown in yeast extract and YM medium with different carbon sources. The cultures were propagated for 72hrs after which enzyme activities were determined as described in Methods. The highest *in vitro* enzyme activity was observed when growth was on yeast extract medium with 2% glucose (table 6).

Table 6

Activity of *t*-cinnamic acid decarboxylase in GRF 18 grown in minimal and complete media with different carbon sources

Medium	carbon source	specific activity [nM styrene/mg dm ³ h]	relative activity
Yeast extract	glucose (2%)	3.7	100.0
Yeast extract	glycerol (3%)	1.3	35.5
Yeast extract	ethanol (4%)	2.5	65.5
Minimal	glucose (2%)	2.9	77.0

The time course of enzyme expression during growth of *S. cerevisiae* GRF 18 in a batch fermentation was studied next. The culture was grown in a 10l stirred tank reactor with a yeast extract basal medium. The following parameters of the culture were measured: $p\text{O}_2$, pH, glucose concentration, dry mass, OD_{600} , enzyme activity, as well as the O_2 and CO_2 concentration in the effluent gas stream. The results are plotted in figure 32.

After inoculation, the culture rapidly commenced exponential growth. Glucose was consumed at approximately 6.3 hours culture time, after which the culture grew linearly after a short lag period. The specific activity of *t*-cinnamic acid decarboxylase displayed an exponential increase which roughly paralleled the growth curve during growth on glucose. As soon as all glucose was consumed, however, the specific activity doubled within an

hour. Specific activity persisted at this level for a further 4 to 5 hours, after which it dropped to a level similar to the one determined at the end of the exponential growth phase. The doubling of specific activity coincides with the first pH minimum during batch growth; whereas the duration of the plateau of high-level specific activity is as long as the phase in which the culture pH becomes more alkaline. It was not possible to unequivocally conclude that the enzyme was expressed constitutively, because the presence of an inducer in the complete medium could not be completely excluded. It could not be concluded from these results whether the rapid rise in specific activity was caused by the complete consumption of glucose or by the minimum in pH or by another parameter.

Figure 32

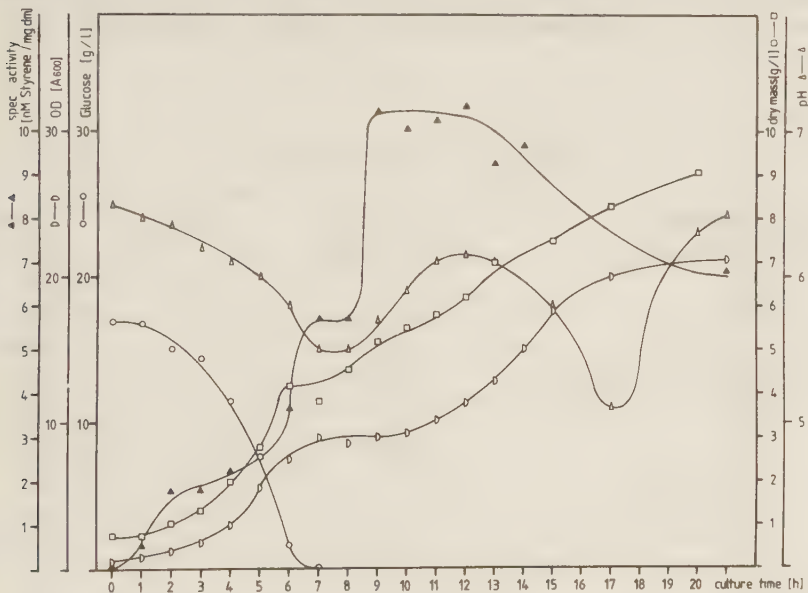


Figure 32 shows the time course of a batch fermentation of GRF 18 in a yeast extract medium. The fermenter was inoculated with 20% by volume of a GRF 18 preculture grown to saturation in the same medium. The following parameters of the culture were measured: pO_2 , pH , glucose concentration, dry mass, OD_{600} , enzyme activity, as well as the O_2 and CO_2 concentration in the effluent gas stream. The RQ was calculated from the QO_2 and QCO_2 , it never rose above $RQ = 1$ (not shown). The pO_2 of the culture never fell below 70% during growth.

The influence of glucose on the expression of the gene was investigated next. GRF 18 transformed with pPD150 was propagated in minimal medium supplemented with L-histidine with glucose as carbon source. In the first phase of this experiment, glucose was added to 0.4% final concentration. After the carbon source had been totally consumed, the culture was starved in respect to energy source for three hours. Glucose was then added to 2% in order to investigate a possible glucose repression.

The results (figure 33) gave no indication of glucose repression. Specific activity was highest immediately after inoculation, this reflected the amount of enzyme in the cells carried over from the preculture. Specific activity of *t*-cinnamic acid decarboxylase did not increase exponentially during the first phase of growth on glucose, as observed in the previous experiment. After the addition of glucose to 2%, the specific activity of the enzyme remained practically constant over a period of three hours.

Figure 33

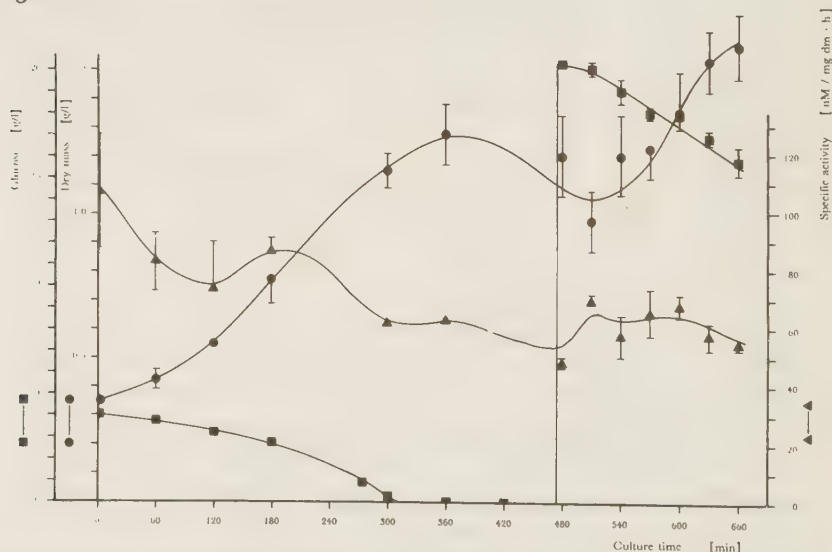


Figure 33 shows the time course of a batch fermentation of transformed GRF 18 grown under selective conditions in a minimal medium supplemented with L-histidine. The total culture volume was 1l. The fermenter was inoculated with 20% by volume of a fresh GRF 18 preculture grown to saturation in the same medium. The following parameters of the culture were measured: glucose concentration, dry mass and enzyme activity. All measurements were performed in duplicate.

The expression of the *CAD1* gene in *petites* was studied next. All previous observations that ρ^- yeast displayed *cad^-* phenotype had been made in *in-vivo* experiments. In the following experiment, *in-vitro* enzyme assays of *t*-cinnamic acid decarboxylase were made with *petite* *S. cerevisiae* cells. After a two-hour incubation of GRF 18 cells with ethidium bromide, performed as described in Methods, respiratory deficient cells were selected on YGG plates. Five ρ^- clones were picked along with one ρ^+ clone which survived the ethidium bromide treatment as *grande*. Untreated GRF 18 and z0 42 were used as controls. The clones were grown to saturation in complete medium, lyophilized and assayed as described in Methods. The results of the *in-vitro* assay are compiled in table 7. The results extend the previous observations made in *in-vivo* experiments to *in-vitro* experiments. They allow the firm conclusion, that no *t*-cinnamic acid decarboxylase activity is present in ρ^- yeast cells.

Table 7

CAD activity in ρ^- strains

Strain	relevant marker	specific activity [pM/h·mg dm]
GRF 18-1	ρ^- , <i>CAD1</i>	0.0
GRF 18-2	ρ^- , <i>CAD1</i>	0.0
GRF 18-3	ρ^- , <i>CAD1</i>	0.0
GRF 18-4	ρ^- , <i>CAD1</i>	0.0
GRF 18-5a	ρ^- , <i>CAD1</i>	0.0
GRF 18-5b	ρ^+ , <i>CAD1</i>	3925.0
GRF 18	ρ^+ , <i>CAD1</i>	4413.0
z0 42	ρ^+ , <i>cad1</i>	565.0

Table 7 is a compilation of the results of *in-vitro* enzyme assays performed with either *petite* or *grande* *CAD1* and *cad1* yeast cells. Strain GRF 18-5b was a clone picked after ethidium bromide mutagenesis, which remained respiratory proficient.

I determined next whether the absence of enzymatic activity was transcriptionally or translationally regulated. Total RNA was prepared (as described in Methods) from ρ^+ and ρ^- *CAD1* cells (GRF 18), as well as from transformed *S. pombe* (see below) and from induced and uninduced *F. oxysporum*. The RNA samples were separated as described in Methods and blotted onto nylon filters. The samples were applied in duplicate, one filter

was probed with single stranded pPD134, the other filter was probed with single stranded pPD133. Hybrids that survived the stringency washes were detected with a colorimetric reaction.

A faint signal was observed only with the RNA sample originating from the ρ^+ *CAD1* strain (results not shown). This was only the case when pPD134 was employed as probe. All other samples did not hybridize with the probes. I conclude that the mitochondrial influence on the regulation of *CAD1* gene expression occurs at the level of transcription.

Gene expression in a heterologous system

The expression of the *CAD1* gene in heterologous systems was studied next. *S. pombe*, which does not possess an endogenous enzyme catalyzing the decarboxylation of *t*-cinnamic acid to styrene, was transformed with pPD150 as described in Methods. The selection of transformed cells was based on the complementation of the *leu 1-32* marker by the *S. cerevisiae* *LEU2* gene carried by pPD150. Transformants were required to grow on minimal medium not supplemented by L-leucine. Transformed cells were picked, purified by streaking on selective medium and then grown to saturation on liquid EMM2 medium. The *S. pombe* cells were then treated exactly as *S. cerevisiae* cells for the *in-vitro* assay of *t*-cinnamic acid decarboxylase. Enzyme assays were performed and analyzed as described for *S. cerevisiae*.

Figure 34

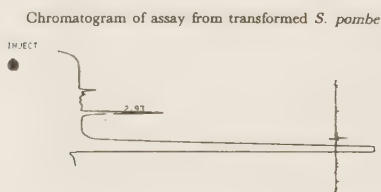


Figure 34 shows an HPLC chromatogram of an assay with lyophilized *S. pombe* cells transformed with pPD150. The trace proceeds from top to bottom; the void volume peak is followed by the styrene peak at 2.93 minutes, the solvent peak is last. Integration was inactivated except for a time window when styrene was expected.

Figure 34 shows a HPLC chromatogram of styrene extracted from an enzyme reaction performed with *S. pombe* cells. The amount of enzyme activity expressed in different clones transformed with the same plasmid showed a large degree of variance (see figure 35). The absolute amount of enzyme activity observed was considerably lower than in untransformed, wild type GRF 18. In figure 35, the results obtained from each HPLC measurement are shown separately and not as usually, as the mean of at

least two measurements. Whether the observed variance was inherently

due to the vector or caused by the insert present in pPD150 was not determined. I conclude that the *S. cerevisiae* *CAD1* gene is expressed in *S. pombe*, albeit not with the same degree of efficiency and/or stability.

Figure 35

Cinnamic acid decarboxylase activity in transformed *S. pombe*

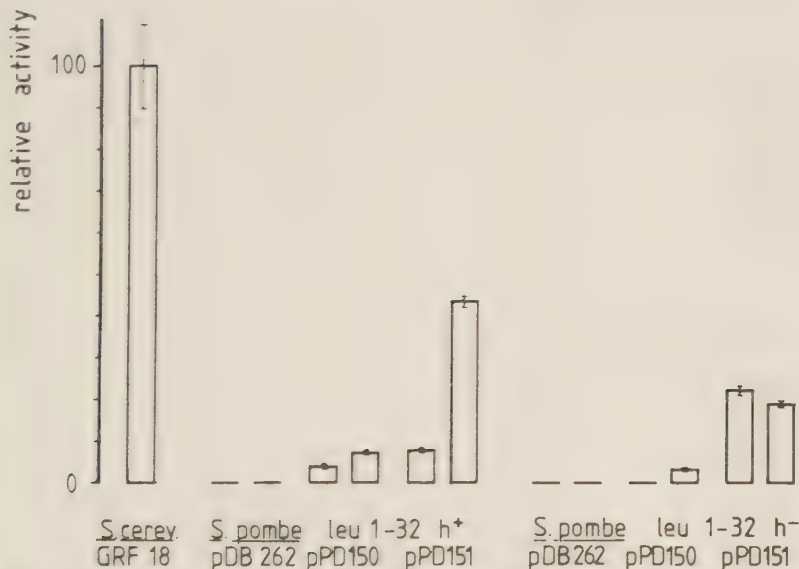


Figure 35 is a compilation of the enzyme activities of *t*-cinnamic acid decarboxylase measured in individual clones of transformed *S. pombe*. For comparison, the activity of untransformed wild type GRF 18 is shown left. The specific activity of the enzyme observed in the reactions was normalized to arbitrary, dimensionless units, with the control set to 100. The error bars represent the limits of the standard deviation from the mean observed in the HPLC measurements.

The expression of *CAD1* in *E. coli* was determined next. *E. coli* strain DH 1 transformed with different plasmids rescued from yeast was streaked out on growth medium either lacking *t*-cinnamic acid or with different concentrations of *t*-cinnamic acid added (table 8). Selective conditions for cells harboring plasmids were imposed by the inclusion of ampicillin in the medium. After one or two days of incubation, growth was scored (table 8).

Table 8

Growth of *E. coli* transformed with plasmids rescued from yeast

<i>E. coli</i> DH 1 with plasmid #	Phenotype of <i>cad</i> ⁻ yeast with same plasmid as DH 1	A	B	Medium C	D	E
A 24 hours growth at 37°C						
pPD 3	<i>cad</i>	+++	+++	++	-	-
pPD 5	<i>cad</i>	+++	+++	+++	-	-
pPD 14	<i>CAD</i>	+++	+++	+	-	-
pPD 19	<i>CAD</i>	+++	+++	+	-	-
pPD 27	<i>cad</i>	+++	+++	+++	-	-
pPD 39	<i>cad</i>	+++	+++	++	-	-
pPD 40	<i>CAD</i>	+++	+++	+	-	-
pPD 48	<i>CAD</i>	+++	+++	+	-	-
pPD 70	<i>CAD</i>	+++	+++	+	-	-

B 48 hours growth at 37°C

pPD 3	<i>cad</i>	+++	+++	+++	++	-
pPD 5	<i>cad</i>	+++	+++	+++	++	-
pPD 14	<i>CAD</i>	+++	+++	++	+	-
pPD 19	<i>CAD</i>	+++	+++	++	+	-
pPD 27	<i>cad</i>	+++	+++	+++	++	-
pPD 39	<i>cad</i>	+++	+++	++	++	-
pPD 40	<i>CAD</i>	+++	+++	++	+	-
pPD 48	<i>CAD</i>	+++	+++	++	+	-
pPD 70	<i>CAD</i>	+++	+++	++	+	-

Basal medium is LB with 50 µg / ml ampicillin

A	without <i>t</i> -cinnamic acid
B	with 1 mM <i>t</i> -cinnamic acid
C	with 2.5 mM <i>t</i> -cinnamic acid
D	with 5 mM <i>t</i> -cinnamic acid
E	with 10 mM <i>t</i> -cinnamic acid

Legend:

+++	Growth of colonies in last of serial streaks
++	Growth of colonies in penultimate of serial streaks
+	Growth of colonies only in primary streak
-	No growth

The results of this experiment are summarized in table 8. Surprisingly, those *E. coli* clones harboring plasmids not conferring a cad^+ phenotype onto yeast transformants when introduced into a *S. cerevisiae cad1* strain, grew better than those clones harboring a plasmid containing the *CAD1* gene. I conclude that the yeast *CAD1* gene is expressed in *E. coli*. However, its expression showed deleterious effects on this host when it is grown on medium containing *t*-cinnamic acid.

3.3.3. Fusions of *CAD1* to *lacZ*

Gene fusions are a powerful tool for the analysis of the expression and regulation of a gene. One of the tools most often utilized as reporter gene is the β -galactosidase structural gene of *E. coli*, *lacZ*. The biochemical assay of this enzymatic marker is not only facile but also extremely sensitive, levels of β -galactosidase as low as 0.001% of wild type have been detected in *E. coli* (Silhavy 1985). Further to this property, other reasons exist why *lac* fusions have often been employed: removal or replacement of up to 27 amino acids from the N terminal region results in little or no effect on enzyme activity (Accolla 1976 and Brickman 1979); *lac*⁻ and *lac*⁺ phenotypes can be selected for in *E. coli* and there are several, nonselective indicator substances for plate assays (Beckwith 1978 and Silhavy 1984). When properly fused to the regulatory elements or to the N-terminus of a yeast gene, the *E. coli lacZ* gene is expressed in *S. cerevisiae* (Rose 1981 and Guarente 1981).

In this work, an *in-vivo* approach was used to generate fusions between the yeast *CAD1* gene and the *lacZ* gene (Rose 1983 and Berman 1984). First, the 5.1kb *Hind*III fragment containing the *CAD1* gene was cloned in both orientations into the vectors YEpZ36 and YEpZ100 (Oberto 1985) upstream of the *lacZ* gene, resulting in the plasmids pZYA to pZYD. These plasmids can replicate in *E. coli* and in *S. cerevisiae* and can be selected for by complementing either a *leuB* mutation in the former or a *leu2* mutation in the latter host or by their ability to confer Ap^r phenotype on *E. coli*. The vectors carry a *lacZ* gene truncated at the 5' end and cannot express β -galactosidase due to the absence of a promoter and a methionine initiation codon. The *lacZ* gene can be activated by an in-phase fusion to another gene possessing its own promoter (Guarente 1980). YEpZ100 lacks an in-phase ATG codon approximately 120bp upstream of the coding sequence of *lacZ* which is still present in YEpZ36 (Oberto 1985). This ATG is not the original initiation codon of *lacZ* in *E. coli*.

The plasmids described above (pZYA to pZYD) were introduced into a recombination proficient *E. coli* strain which constitutively expresses *lacY* but completely lacks an endogenous *lacZ* gene. Fusions were then generated *in-vivo* by deleting intervening DNA sequences (details of the procedure are described in Methods). Clones harboring *in-vivo* deleted plasmids were selected by requiring *E. coli* AMA 1004 to grow on lactose as sole carbon source. After purifying these clones by streaking, their plasmids were isolated and analysed by restriction enzyme digestion. In order to preclude further rearrangements events, plasmids were then introduced in-

to a recombination deficient *E. coli* strain which constitutively expresses *lacY*, before β -galactosidase activity was assayed.

A total of 298 *lac*⁺ colonies were analysed. Clones transformed with pZYA or pZYB (constructed with YEpZ36) gave rise to *lac*⁺ colonies at a much greater frequency than those harboring pZYC or pZYD. Restriction analysis of plasmids isolated from *lac*⁺ clones originally transformed with pZYA or pZYB revealed that in all plasmids, only short stretches of DNA from the ends of the 5.1kb insert had been eliminated. The presence of an initiation codon downstream of the *Hind*III site used for insertion of the 5.1kb fragment and 120bp upstream of the coding sequence of the truncated β -galactosidase gene on YEpZ36, imposed less rigorous requirements on sequences selected for their capacity to direct transcription of the distal *lacZ* gene. None of the deletion plasmids deriving from pZYA and pZYB were studied further.

After the initial purification step, 125 clones with a *leu*⁺ and Ap^r phenotype were identified which harbored deletion plasmids derived from pZYC or pZYD. A one-enzyme restriction analysis of plasmids prepared from these clones was performed next. The information was adequate to identify and discard siblings with identical deletions that had been generated by the same event during growth in the liquid culture. Only one of these plasmids was then analyzed further.

Restriction analysis with *Eco*RI, *Bam*HI and *Hind*III was then performed to determine the extent of the deletion and to localize the junction. A large number of the plasmid preparations were inhomogenous at this stage. This was concluded from the pattern of restriction fragments observed in the gels. Fragments not fluorescing proportional to their molecular weight when compared to the other fragments present in the same lane were assumed to belong to a further, distinct plasmid species. This mixture was purified by retransforming *E. coli* and analyzing the plasmids prepared from individual transformants by restriction enzyme digestion. Many plasmids derived from inhomogenous populations were incapable to simultaneously confer Ap^r, independence from L-leucine supplementation and to replicate autonomously. These plasmids were not further analyzed.

A total of 30 plasmids derived from either pZYC or pZYD were finally obtained and studied in more detail. Of these, 14 had deletion end points within the 5.1kb *Hind*III fragment, whereas the the junctions of the other 16 plasmids were further upstream, in the yeast *LEU2* gene. Of the latter 16 plasmids, 11 still complemented the *leuB* mutation in *E. coli*. The

Figure 36

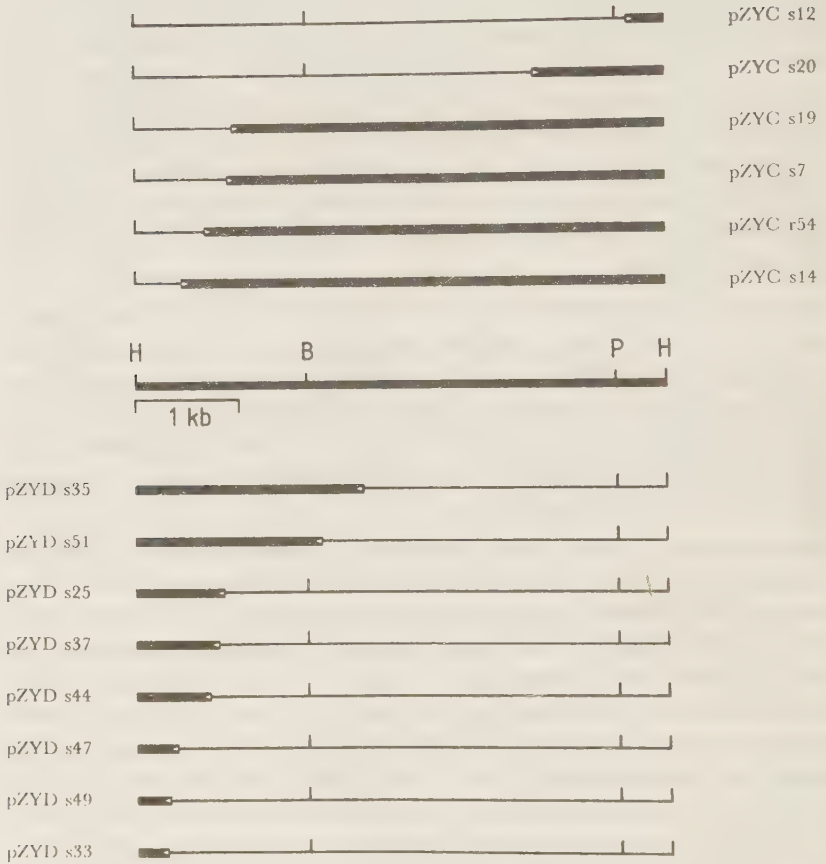
Junctions of *in-vivo* deleted *CAD-lacZ* Fusions

Figure 36 shows the junctions between DNA sequences originating from within the 5.1kb *Hind*III fragment and the truncated *lacZ* gene present on plasmid YEpZ100. In the upper and lower part of this panel, the thin line represents the remaining insert DNA, whereas the thick line symbolizes the vector sequence. The arrowhead in the thick line at the junction point shows the direction of transcription of the *lacZ* gene. The 5.1kb *Hind*III fragment is inserted in opposite orientations in the progenitor plasmids pZYC and pZYD respectively.

orientation of the inserts and the deletion end points of the former 14 plasmids are shown in figure 36.

The enzyme activities expressed from these deletion plasmids with *lac* fusions were determined in *E. coli* PDC 75 and in *S. cerevisiae* z042 by the procedures summarized in Methods. In *E. coli*, only β -galactosidase activity was assayed, whereas in *S. cerevisiae* both β -galactosidase activity and *t*-cinnamic acid decarboxylase activity were assayed. The results from some assays were subjected to statistical analysis with Student's *t*-test as described in Methods. The results are summarized in table 9.

When assayed in *E. coli* PDC 75, the β -galactosidase activity encoded by transcripts originating from within the 5.1kb *Hind*III fragment was observed to be higher than the activity observed in the uninduced control DH 1. This β -galactosidase activity was furthermore at least 100-fold higher than the residual activity observed from the non-deleted parental plasmids pZYC and pZYD.

In *S. cerevisiae* z042, the levels of β -galactosidase activity observed were very low. The plasmid responsible for the highest level of expression in *E. coli* (pZYCs7) exhibited the second highest expression level in yeast. The β -galactosidase activity observed in nine yeast transformants differed significantly from the negative control (which was the average of the activity determined for z0 42 transformed with pPD150, pZYC and pZYD) at 0.99 confidence level.

The levels of activity of *t*-cinnamic acid decarboxylase observed in *S. cerevisiae* z042 transformed with the fusion plasmids allowed their classification into three distinct groups. The first group, comprised of plasmids responsible for high levels of enzyme activity of a magnitude similar to the positive controls (pZYC and pZYD), consisted of those plasmids with only short stretches of the insert deleted (pZYCs12 and pZYCs20; pZYDs25, pZYDs37, pZYDs44, pZYDs47, pZYDs49 and pZYDs33). The second group was comprised of plasmids responsible for levels of enzyme activity not significantly different from the negative control (pZYDs41, which is one of the *LEU2* - *lacZ* fusions completely lacking any 5.1kb *Hind*III fragment derived sequences). The plasmids pZYCs19 to pZYCs14 and pZYDs35 belong to this group. The third group with an intermediate level of activity had only one member: pZYDs51.

From the data presented in table 9 and figure 37, I conclude that a *CAD1* - *lacZ* fusion exists in plasmids pZYDs51, pZYDs25, pZYDs37

Table 9

Enzyme activities expressed from fusion plasmids in *E. coli* and *S. cerevisiae*

Plasmid or strain	β -Galactosidase activity in <i>E. coli</i> PDC 75				β -Galactosidase activity in <i>S. cerevisiae</i> z0 42				CAD activity in <i>S. cerevisiae</i> z0 42			
	Units [nM oNP/min OD ₆₀₀]	% SD	n	relative to DH 1 (%)	Units [pM oNP/min $\cdot$ mg dm]	% SD	n	relative to best (%)	specific activity [pM/h $\cdot$ mg dm]	% SD	n	relative to wt (%)
pZVC s12	2.37	23.0	8	0.6	5.2	30.0	4	0.2	53213.0	6.8	4	97.4
pZVC s20	5.44	56.0	8	1.5	41.2	42.0	4	2.2	45324.0	6.9	4	82.9
pZVC s19	21.98	9.0	12	6.1	1886.0	15.0	4	100.0	1399.0	20.0	4	2.6
pZVC s7	181.11	2.6	8	50.0	289.0	10.0	4	15.3	1982.0	48.0	4	3.6
pZVC r54	136.89	6.4	8	38.0	1.1	67.0	4	<0.1	1135.0	82.0	4	2.1
pZVC s14	5.93	12.6	16	1.6	3.3	15.0	4	0.2	965.0	13.0	4	1.8
pZYD s35	55.33	7.2	8	15.0	1.2	29.0	4	<0.1	1245.0	7.8	4	2.3
pZYD s51	122.89	7.8	8	34.0	1.7	16.0	4	0.1	4938.0	13.0	4	9.0
pZYD s25	10.33	53.0	8	2.9	0.6	22.0	4	<0.1	44245.0	6.9	4	80.9
pZYD s37	8.80	12.0	8	2.4	8.7	20.0	4	0.5	33431.0	4.4	4	61.2
pZYD s44	9.11	6.6	8	2.5	2.2	12.0	4	0.1	35633.0	6.2	4	65.2
pZYD s47	2.69	11.0	8	0.7	0.6	9.6	4	<0.1	33446.0	4.8	4	61.2
pZYD s49	26.67	18.0	8	7.4	0.4	31.0	4	<0.1	48680.0	18.0	4	89.1
pZYD s33	9.13	46.0	8	2.5	0.7	8.2	4	<0.1	35066.0	12.0	4	64.2
DH 1 induced	361.56	20.0	8	100.0	nd	nd	0	nd	nd	nd	0	nd
DH 1 not induced	2.24	2.7	4	0.6	nd	nd	0	nd	nd	nd	0	nd
PDC 75 induced	0.00	100.0	4	0.0	nd	nd	0	nd	nd	nd	0	nd
PDC 75 not induced	0.00	40.0	4	0.0	nd	nd	0	nd	nd	nd	0	nd
pZ100	0.00	0.0	4	0.0	nd	nd	0	nd	nd	nd	0	nd
pZYD s41	nd	0.0	nd	nd	0.8	20.0	4	<0.1	1492.0	15	4	2.7
pPD 150	nd	0.0	nd	nd	0.7	66.0	4	<0.1	30259.0	7.1	4	55.4
pZVC	0.02	82.0	4	0.0	0.3	17.0	4	<0.1	53854.0	14.0	4	98.5
pZYD	0.02	100.0	4	0.0	0.3	0.0	4	<0.1	55467.0	21.0	4	102.0

and pZYDs44. This conclusion independently corroborates the result obtained in 3.3.1. that the coding strand for the *CAD1* gene is the one in which the recognition sites for the following restriction enzymes appear in the order: 5' *Hind*III *Pvu*II *Bam*HI *Kpn*I *Hind*III 3'.

An independent test of this conclusion was performed. If a *CAD1-lacZ* fusion exists in plasmids pZYDs51, pZYDs37 and pZYDs44 and the mRNA of a putative fusion protein is transcribed under the control of the *CAD1* regulatory elements, then both β -galactosidase activity and *t*-cinnamic acid decarboxylase activity should be regulated in an identical manner. I intended to test this prediction by assaying enzyme levels in *petites* transformed with this and with control plasmids. Under these conditions neither β -galactosidase activity nor *t*-cinnamic acid decarboxylase activity should be observable.

Figure 37



Figure 37 is a photograph of the plates onto which transformants, which had been mutagenized with ethidium bromide for two hours, had been spread. On each of the plates visible on the top row, *grandes* and *petites* are visible. The yeast cells plated on the top row were transformed (from left to right) with pZYC s19, pZYD s35 and pZYD s51 respectively. The yeast cells plated on the middle row had harbored the following plasmids (from left to right): pZYC, pZYD, pZYC s20, pZYD s37 and pZYD s44. The bottom row shows (from left to right): GRF 18 5a, a *petite*; GRF 18, a *grande*; they were employed as negative and positive controls for the plates. The following two plates, divided into four sectors each, are dilution streaks of all of the transformants tested in this experiment on the same plates before they were mutagenized by ethidium bromide.

Two fresh transformants of each plasmid were grown under selective conditions to middle exponential phase. These cultures were then mutagenized with ethidium bromide as described in Methods and plated onto YM. However, as shown in figure 37, none of the transformants harboring a plasmid belonging to the group responsible for high level expression of *t*-cinnamic acid decarboxylase survived the procedure. A repetition of the experiment yielded the same result. Because controls were unavailable, the prediction that a fusion protein is expressed in pZYDs51 and that the transcription of this hybrid mRNA is under the control of the *CAD1* regulatory elements could not be tested.

4. Discussion

4.1. How does *t*-cinnamic acid inhibit growth of *CAD* yeast?

4.1.1. Summary of results

The results presented in 3.1.1. show that *CAD* yeast cells are inhibited in their growth by *t*-cinnamic acid. The inhibition is *pH* dependent and increases as *pH* is lowered. The growth inhibition is alleviated by the presence of glucose in the medium. Furthermore, *t*-cinnamic acid interferes with mass flux across the membrane.

4.1.2. Discussion

The experiments described in 3.1.1. had been designed to show why *t*-cinnamic acid inhibits growth of *S. cerevisiae* and how this effect is mediated.

It is well known that lipophilic acids exert a *pH* dependent growth inhibitory effect on yeast cells, which is magnified as the *pH* is lowered. Baranowski *et.al.* (1980) have shown that *S. cerevisiae* is inhibited by naturally occurring hydroxycinnamic acids. However, there are contradicting reports regarding their mode of entry into the cell as well as their mode of inhibition. The literature discussed below deals with benzoic acid, the conclusions drawn from work with this substance should also be applicable to *t*-cinnamic acid. However, *t*-cinnamic acid is metabolized by yeast, while benzoic acid is not.

Most authors (Cramer 1977, De La Peña 1982, Krebs 1983) agree that equal intra- and extracellular concentrations of the undissociated species are attained by passive diffusion; whereas Macris (1975) shows evidence for an uptake system and facilitated diffusion as mechanism. The experiments in this work were not designed to discriminate between these proposals so that the results obtained in this work do not bear on this issue.

For benzoic acid, two different mechanisms for the growth inhibitory action have been proposed: Freese *et.al.* (1973) claim inhibition of substrate transport into the cells as decisive; Krebs *et.al.* (1983) propose that intracellular acidification is responsible, caused by the large amounts of protons liberated when the newly entered undissociated acid equilibrates as described by the Henderson-Hasselbach equation. The marked sensitivity of phosphofructokinase to a decrease in *pH* then is believed to result in an inhibition of glycolysis.

The results of this work show that the sheer number of protons liberated into the intra cellular space is not decisive, because growth inhibition should then be more pronounced at higher rather than at lower *pH* values. The alleviation of growth inhibition caused by the addition of glucose to the medium, which can be interpreted as the ability to cope with this import of protons, has been described for the first time in this work. It cannot be deduced yet from the results, whether the glucose consumed has to come up for inefficiencies in glycolysis caused by adverse *pH* conditions or whether the additional ATP is hydrolyzed in proton export or is spent on energy dependent transport processes.

The results of the experiments involving *p-fluoro* phenylalanine allow the conclusion, that *t*-cinnamic acid influences the molecular order of the lipid bilayer. As no other uptake mechanism for L-phenylalanine is known (Cooper 1982), synergistic effects in growth inhibition point to an enhanced transverse penetration of *p-fluor* phenylalanine by other means.

The difficulties encountered in the determination of the internal *pH* of yeast cells with spin probes in the epr spectrometer can be explained by the rapid reduction of radicals in living cells (Mehlhorn 1982). An experimental approach not involving radical spin probes, such as ^{13}C and ^{31}P NMR measurements of *pH* dependent changes in the chemical shifts of the vinyl protons of *t*-cinnamic acid and of the protonation of ATP, respectively should make this determination possible.

4.1.3. Summary and Conclusions

The growth of yeast is inhibited by *t*-cinnamic acid. I have shown that this inhibition is caused by interference with cellular energy metabolism as well as with transport across the cytoplasmic membrane. It can be concluded from this work, that the models proposed by Krebs *et.al.* (1983) and Freese *et.al.* (1973) are insufficient to explain the observed effects. The individual contribution of each observed effect to overall growth inhibition remains to be assessed after determination of intracellular *pH* levels and of the amounts of undissociated acid dissolved in the membrane.

4.2. Is the decarboxylation of *t*-cinnamic acid and its analogs the authentic enzymatic function of the *CAD* gene?

4.2.1. Summary of results

The results presented in 3.1.2. prove that *S. cerevisiae* decarboxylates *t*-cinnamic acid stereospecifically to styrene. Analogs that occur naturally as intermediates in the phenylpropanoid pathways of higher plants were decarboxylated to the corresponding substituted styrenes. Substitutions at the 3- and 4- positions of the aryl moiety with a nitro group did not abolish activity. Some substances with carboxyl functions decreased the enzymatic activity when added to the assay. 3-phenylpropionic acid and 2-phenylethanol were identified as potent inhibitors. The results presented in 3.1.3. show that CAD activity is not restricted to *S. cerevisiae* alone: *Fusarium oxysporum* shows CAD activity, whereas *Schizosaccharomyces pombe* does not.

4.2.2. Discussion

It has to be emphasized that all enzymatic assays were done with lyophilized cells. Untreated yeast cells completely exclude inert probing molecules with a molecular weight over 620. This sieving can be ascribed to the cell wall, whereas the uptake-exclusion threshold for the cell membrane with inert probing molecules lies at MW = 110 (Scherrer 1974). However, the permeability of yeast cells increases dramatically when dehydrated below 20% relative humidity. This phenomenon is not reversed rapidly by subsequent rehydration (Rapoport 1982). This observation can easily be explained by the collapse of membrane integrity as the hydrophobic interactions decrease, when water, the polar solvent predominantly stabilizing them, is withdrawn. Freeze dried cells thus can be legitimately used in assays involving permeation of molecules larger than MW = 110.

Substrates

The results of this work suggest that the decarboxylation of phenylacrylic acids to styrene and its analogs is the true enzymatic function of the enzyme from *S. cerevisiae*. Only phenylacrylic acids as substrates give rise to products, no other products were ever found when other substances were added to the assays.

The results of this work show that cinnamic acid decarboxylase utilizes the naturally occurring substrates with different efficiencies. In terms of total

yield of product, substrate specificity was in the following order: ferulate > *p*-coumarate > *t*-cinnamate (1: .84: .79). In an *in vivo* assay, Goodey and Tubb (1982) observed the following order: ferulate > *t*-cinnamate > *p*-coumarate (1: .51: .33). Wackerbauer *et.al.* (1978) found: *p*-coumarate > ferulate; whereas Albagnac (1975) found: *t*-cinnamate > ferulate > *p*-coumarate. The results in the latter three papers were obtained with *in vivo* assays using different growth media and different yeast strains. The differences observed in substrate specificity probably reflect these differing experimental protocols. It may be a consequence of the *in-vivo* assay for example, that the substrates entered the cells with different efficiencies. It will only be possible to reconcile these conflicting results when the assays are performed with purified enzyme.

Other authors have not observed any decarboxylation product in assays with caffeic acid. A possible explanation may be inadequate instrumental sensitivity with GC. 3,4 *diOH*-styrene was detected in assays performed with caffeic or with chlorogenic acid, the spectrum was taken from the product of the latter due to a slightly higher amount. It was assumed that the caffeic acid in the latter assay was liberated by spontaneous hydrolysis of chlorogenic acid rather than by an esterase activity of *S. cerevisiae*.

Phenylacrylic acids are key intermediates in plant secondary metabolism, leading to diverse natural products such as lignin, flavonoids, isoflavonoids, coumarins, lignans and phytoalexins (Goodwin 1983). A number of these products are involved in disease resistance mechanisms that have evolved in higher plants (Legrand 1983; Kolattukudy 1983; Bell 1981; Ryan 1983; Darvil 1984). It has been demonstrated that some naturally occurring phenylacrylic acids directly exhibit antimicrobial activity: Stahmann *et.al.* (1976) have shown that the germination of coffee rust uredospores is inhibited by cinnamic acid derivatives and Gupta *et.al.* (1976) identified ethyl *p*-methoxycinnamate as the main antifungal agent from the East Indian plant *Curcuma zedoaria*.

The ability to eliminate these growth inhibitory compounds should confer a selective advantage on an organism utilizing plant material as substrate. To my knowledge CAD or CAAD activity has been described only in plant pathogens or saprophytic organisms living on matter rich in plant phenolics. The wild habitat of yeast is decaying fruit and vegetable matter, whereas *F. oxysporum* is a plant pathogen. *Enterobacteriaceae* inhabiting the guts of herbivores or omnivores are also confronted with phenylacrylic acids. However, the non-reductive decarboxylation of these acids is not the

only strategy for elimination: *S. pombe*, which was originally isolated from East African millet beer (Mitchison 1970) and thus would classify as saprophyte, does not show CAD activity. Phenylacrylic acids are presumably degraded by a different pathway in this organism, probably by reduction in the vinyl region. This is not surprising because it has been demonstrated in the last few years that *S. pombe* is only very distantly related to *S. cerevisiae*. This can be judged by taxonomic criteria: biochemical organization of the L-tryptophan biosynthesis pathway, (Schweingruber 1973); by the comparison of: 5S RNA (Mao 1982) or cytochrome *c* (Russel 1982) sequences; by the comparison of: *ras* protein functions, cell cycle regulation, regulation of gene expression and the status of ARS elements (autonomously replicating sequences) (Russel 1986).

Any involvement of *t*-cinnamic acid decarboxylase of *S. cerevisiae* in other metabolic reactions or pathways can probably be excluded. Bacterial amino acid decarboxylases are highly expressed with kinetics similar to those observed in 3.2.4.. Their physiological role is to regulate the pH of the culture media when acidification occurs late in growth (Sukhareva 1986). However, the absence of any stimulation of enzymatic activity in assays performed with the addition of either thiamine pyrophosphate, pyridoxal phosphate or pyruvate, which are essential cofactors in amino acid decarboxylation, makes it very improbable that CAD is involved in this type of reaction.

None of the intermediates of the tricarboxylic acid cycle examined showed any influence on enzymatic activity. It is highly improbable then, that CAD is involved in this cycle.

Inhibition in the presence of chelating compounds

The inhibition of enzymatic activity observed when compounds with chelating potential were present in the assay cannot be easily explained. The general inhibition by anions as shown by Fridovich (1963) for acetoacetate decarboxylase is a possible explanation. At the optimal pH, the charged, ionic form of *t*-cinnamic acid is 35 – fold more abundant than the undissociated form. A stable binding interaction between substrate and enzyme would only be possible if charge repulsions were excluded. A positively charged residue in the vicinity of the active site could shield the negative charge of the substrate and thus stabilize the binding interactions. If the site of substrate binding is not buried deep inside the protein, a negatively charged moiety could be sufficient to compete with the substrate molecule for binding at this site.

Another possible explanation is a general inhibition by carboxyl groups. This explanation can be viewed as a restricted version of the above proposal. If steric constraints are more severe than assumed above, only carboxyl anions may compete for binding. Citrate, which was tested as buffer, as well as CDTA and EGTA, however, not only possess carboxyl functions but also chelating capabilities. It is therefore conceivable that the inhibition observed is due to the chelating capabilities of these substances. In this model, bivalent metal cations would act as a charge stabilizing bridge between a negatively charged residue of the protein and the anionic carboxyl function.

However, calculation of the conditional stability constants of the cation-CDTA complexes as described by Inczedy (1976) reveals a striking parallelism to the observed pattern of inhibition. The CDTA-Mg²⁺ complex has the lowest stability constant at pH 6 ($\log \alpha_{\text{Mg}^{2+}} = 4.1$) whereas the CDTA-Co²⁺ complex has a high one ($\log \alpha_{\text{Co}^{2+}} = 12.7$). The conditional stability constants for Zn²⁺ and Cd²⁺ have intermediate values.

The calculated constants, however, can only be approximations. It is impossible to determine the chemical activity for each species in the assay system employed. Cd²⁺ for example, is known to interact with ubiquitous glutathione and other sulfhydryl compounds. The point to be made is: low conditional stability constants correlate with incomplete reversal of inhibition by CDTA, high stability constants correlate with complete reversal and even slight improvement over controls. A coordinately bound cationic species, present at concentrations where all complexing functions are saturated, completely sequesters the available carboxylic functions. Metal-ligand complexes generally assume octahedral or other bulky three-dimensional configurations (Inczedy 1976), and thus cannot compete further with free carboxyl groups at sterically restricted sites.

The inhibition of enzymatic activity observed when compounds with chelating potential were present in the assay at high concentrations in respect to the substrate is therefore probably caused by unspecific inhibition due to freely accessible carboxyl functions. The possible involvement of a cationic counter-ion, stabilizing interactions with the carboxylate anion, however, cannot be completely excluded.

Enzyme-substrate interactions in binding

It is remarkable that there were no significant interferences with enzyme-substrate interactions from molecules missing the planar double bond in

the alkyl moiety with the sole exception of 2-phenylethanol (see below). Conversely, the inhibition of the enzymatic reaction in assays where one of the following substances were added: 2-hexenic acid, 4-phenyl,3-butenic acid or 3-(3-indolyl)-acrylic acid, suggested that the planar double bond is an essential but not the only criterion for successful binding interactions. The absence of interference with styrene evolution when urocanic acid is added to the assay could be explained by the polarity of the imidazolyl moiety distal to the planar double bond. The imidazolyl moiety is a much stronger base ($pK_{30^{\circ}C} = 6.88$) than the indolyl moiety ($pK_{30^{\circ}C} = -3.5$) (Mark 1981). At the pH of the assay, approximately 75% of the urocanic acid molecules will be positively charged at the imidazolyl moiety. This calculation is based on the assumption that influences from the alkenyl moiety on the value of the pK of protonation can be neglected.

I thus propose, that favored substrate-enzyme binding interactions occur with molecules displaying the following features: A carboxyl function with a planar double bond immediately distal (between the α - and β - carbon) to it; adjacent to this unsaturated bond, a non-polar moiety which usually is an aryl ring. It should be rewarding to test this hypothesis on the putatively differential inhibitory effects of 3-hexenic acid *versus* 2-hexenic acid and on 4-phenyl,2-butenic acid *versus* 4-phenyl,3-butenic acid.

The observed reduction of styrene production when 2-phenylethanol is added to the assay can most probably be attributed to membrane alterations brought about by this substance (Bohn 1974). This issue can only be definitely resolved when a purified preparation of enzyme is used.

Enzyme-substrate interactions in the catalytic reaction

The only small degree of interference with styrene evolution when L- α -aminooxy- β -phenylpropionic acid is added to the assay is readily explained. First, this substance lacks the planar double bond found essential for binding interactions. Second, this substance is a transition state analog for the elimination process of phenylalanine-ammonia-lyase (Lienhard 1973). The alignment must be very fine-tuned, so that a similar situation in a molecule catalyzing a completely different reaction can be expected to be highly improbable.

The NMR measurements confirm earlier observations on the stereochemistry of the reaction. Parry (1975) and Gramatica *et.al.* (1981) have performed NMR measurements with 4-OH styrene and a number of other substituted styrenes produced in *in vivo* assays of *Aerobacter aerogenes*

and *Saccharomyces cerevisiae*, respectively. In all cases, the decarboxylation has been found to proceed with retention of the geometry about the double bond.

Parry (1975) and Manitto *et.al.* (1975) have proposed a plausible reaction mechanism for this decarboxylation. They have independently suggested a two step addition-elimination sequence consisting of a *cis*-addition followed by a *trans*-elimination or a *trans*-addition followed by a *cis*-elimination. This mechanism would imply an attack by a nucleophilic residue of the enzyme on the β -carbon atom.

The proposal for a two step addition-elimination reaction sequence can be independently corroborated by results from this work. The observation, that 3-phenylpropionic acid is such a potent inhibitor can be easily explained if one assumes the proposed reaction mechanism to be correct. The enzyme should then be able to open the linear alkynyl triple bond in the initial addition reaction but then be unable to progress further than this intermediate stage, because the energetics of the remaining planar double bond structure exclude the elimination reaction.

It should be very rewarding to synthesize *t*-cinnamic acid labelled with ^{13}C either at the carboxyl function or alternatively at the α or at the β carbon position and then monitor the progress of the decarboxylation reactions *in vitro* in an NMR. 3-phenylpropionic acid labelled at the β carbon position with ^{13}C should provide valuable insights into the reaction mechanism and binding interactions when assayed *in situ* in the NMR. It will be necessary, however, to elucidate the type of inhibition caused by this compound in advance with purified enzyme.

4.2.3. Summary and Conclusions

I conclude that decarboxylation of phenylacrylic acids is the authentic function of the *CAD* gene product of *S. cerevisiae*. The function of this enzyme in the natural habitat of yeast is the detoxification of growth inhibitory compounds produced by higher plants. The enzymatic reaction is inhibited in the presence of carboxylate anions. Inhibition by 3-phenylpropionic acid corroborates previous evidence for a two-step addition-elimination reaction sequence.

4.3. The *t*-cinnamic acid decarboxylase gene is dominant and encoded in the nucleus

4.3.1. Summary of results

Stable, nonconditional and respiratory proficient *cad⁻* mutants were obtained after nitrosoguanidine mutagenesis. The different mutant alleles all belong to one complementation group. The wild type allele is dominant over the mutant alleles tested. The genetic information for *t*-cinnamic acid decarboxylase is encoded in the nucleus.

4.3.2. Discussion

cad⁻ mutants

The results have shown that a small reduction in enzyme activity suffices to reveal *cad⁻* phenotype. This parallels a prior observation of pronounced threshold effects in respect to concentration affecting resistance to *t*-cinnamic acid: None of the wild type yeast tested in this work demonstrated resistance to *t*-cinnamic acid (growth on **MEA**) when the concentration challenging them was elevated by as little as 25% (results not shown).

I have demonstrated that mutants with reduced enzymatic activity are definitely viable. However, as no mutants with more drastic reductions in specific activity were isolated, it was not possible to unambiguously conclude that the *CAD1* gene product is not an essential gene. Recently developed techniques for yeast genetics, however, make it possible to bear on this issue. It is possible to either disrupt a gene by integrative transformation (Rothstein 1983) or to evict a gene (Winston 1983) by recombinative processes, thus completely eliminating the specific gene function. Viable progeny then are conclusive proof of the non-essential function of the gene product.

The observed reversion frequencies are in the magnitude observed for other yeast genes. Prakash and Sherman (1973) have observed spontaneous reversion frequencies of less than 1×10^{-7} revertants per cell at the iso-1-cytochrome *c* locus; van Borstel (1978) finds spontaneous reversion rates between 1×10^{-8} and 4×10^{-8} revertants per cell and generation which, when expressed as frequency and assuming 25 generations, is in the range of 1×10^{-6} and 2.5×10^{-7} revertants per cell. The low reversion frequencies observed for the *cad1* alleles thus allow the conclusion, that the isolation of the *CAD1* allele by complementation is feasible.

Mitochondrial influence on the phenotype

The results obtained from all genetic crosses reveal a mitochondrial influence on the expression of $\text{cad}^{+/-}$ phenotype. The results can be best interpreted by assuming a modulation of the degree of resistance to *t*-cinnamic acid mediated by the mitochondrial species present. Whether this modulation affects enzyme activity or the level of gene expression cannot be clarified at this stage. In order to discuss the topic of mitochondrial influence, a summary of those aspects of mitochondrial genetics in *S. cerevisiae* pertinent to the issue follows.

The entire mitochondrial population of a yeast cell, the chondriome, is composed of approximately 50 molecules of mtDNA. The chondriome is morphologically not a static entity, the number of topologically unlinked organelles can vary between one and fifty per cell (Stevens 1981). Mitochondrial genetics in yeast can be treated best as population genetics at the intracellular level. A model proposed by Dujon *et.al.* (1981) has the following premises: (1) Random pairing and recombination between the different mtDNA molecules of a cell. (2) multiple rounds of pairing and recombination and (3) random segregation. Furthermore, in a mitochondrial cross, the ratio with which the two alleles of a locus are represented in the progeny are the same for all loci concerned (obviously only under non-selective conditions). The coordinated output allele ratio is so characteristic of mitochondrial genes that it can be used as a diagnostic test to determine whether or not an allele is carried by the mtDNA.

The zygote issued from a cross of two nonisomitochondrial parents is heteroplasmic. However, a homoplasmic state is rapidly established. After some 20 to 25 generations, which corresponds to the number of generations in a single colony, virtually all progeny are homoplasmic. Backer *et.al.* (1985) have demonstrated that the combined action of random drift and of random partitioning is the underlying mechanism primarily responsible for the generation of homoplasmic cells under non-selective conditions. For the population as a whole, the frequency of alleles is conserved (if none of the alleles confers an advantage or a disadvantage on the cell harboring it), although it is a random event which specific allele is inherited by a cell.

The extremely high recombination frequency between individual mitochondrial genomes results in the observation that genetic linkage is only apparent for markers physically not separated further than 1000bp. Frequencies of 0.1% wild type recombinants have been observed for markers separated

by not more than 30bp. Therefore, a general mapping procedure to evaluate genetic linkage of a new allele with a known mitochondrial marker is not a useful strategy when intending to locate it physically on the mtDNA.

When ρ^- cells are involved in a cross, the coordinated output allele ratio is skewed. ρ^- clones or *petites* harbor mitochondria with large deletions in their genome. No mitochondrially encoded gene is expressed in these clones. The remaining mtDNA sequences are autonomously replicated, amplified up to hundredfold, resulting in an amount of DNA comparable to the wild type. Zygotes from a cross of ρ^+ with ρ^- strains issue either: only ρ^- diploid cells, or predominantly ρ^+ clones or mixtures of ρ^+ and ρ^- cells. *Petites* of independent origin differ in the degree with which they interfere with the development of the ρ^+ character (Sherman 1983). This property of ρ^- clones, which is termed the "degree of suppressiveness" is defined as the probability that a zygote, generated from the cross of a ρ^+ with a ρ^- strain will give rise to a clone composed entirely of ρ^- cells (Sherman 1983, Nagley 1973).

The results obtained in all genetic crosses reveal a mitochondrial influence on *t*-cinnamic acid resistance. The absence of any recombinant with wild type phenotype restored in the crosses between the different *cad^-* mutants is strong evidence for a nuclear location. If the *t*-cinnamic acid decarboxylase gene were located mitochondrially, intragenic recombination events would be very frequent. With a total of over five thousand clones evaluated in these crosses, recombination would have been detected except for the unlikely case that all mutations in the locus were clustered over a distance of less than 50bp.

The results obtained in crosses between *cad^+* and *cad^-* strains (summarized on the left side of table 2) can be explained best by a mitochondrial species-dependent modulation of the degree of resistance displayed. This effect has also been observed by Tubb *et.al.* (1981). These authors have demonstrated that the replacement of the mitochondrial genome of a *cad^+* strain with one derived from another *cad^+* strain will influence the amount of decarboxylation product formed. In one case, they observed a reduction of 16%, significant enough to make the product undetectable in beer in an organoleptic test.

This mitochondrially mediated effect explains the delayed appearance of some *cad^+* clones and the sensitivity of other clones when challenged with 0.6 mM *t*-cinnamic acid. The combined effects of resistance modulation and sharp sensitivity thresholds would suffice to cause the observed

results even if specific activity were reduced only slightly.

My conclusion, that cad^+ is dominant over cad^- has also been drawn by Goodey *et.al.* (1982). In a genetic analysis performed by dissecting tetrads and scoring the segregation of markers, the authors find that the cad^+ phenotype is inherited as a single dominant marker.

The results presented in table 3 are a further indication of mitochondrial influence on the phenotype displayed by a cell. In these experiments, the progeny of a single zygote were analyzed. Due to the purification step performed before the cultivation in sporulation medium, the diploids committed to meiosis were homoplasmic before the onset of sporulation. Any explanation of the observed frequency of resistant progeny with the suppressiveness of ρ^- mitochondria can be excluded due to the inherent elimination of clones not harboring ρ^+ mitochondria in the course of sporulation.

However, suppressiveness does not preclude recombination between individual mtDNA genomes. On the contrary, the amplification of short sequences in ρ^- clones increases their input allele ratio significantly. Frequently, ρ^- clones exhibit properties termed "effacement" and "high integration frequency" which depend on the residual sequences present. The ρ^+ progeny issued from crosses with these ρ^- clones are virtually all recombinants (Dujon 1981). Respiratory proficient recombinants would not have been identified as recombinants in my experiments. However, these recombinant mitochondria could vary in the degree of resistance displayed.

The ratio of cad^+ to cad^- progeny in the experiments summarized in table 3 can be predicted to be 1:1, assuming a nuclear location of the *t*-cinnamic acid decarboxylase gene. I interpret the large deviations from this ratio observed in these experiments as caused by mitochondrial influences.

In the crosses involving the *cad1-7* and the *cad 1-125* alleles, an influence of the parent strain contributing the wild type allele on the frequency of cad^+ progeny can be seen. It remains to be determined whether this is due to an intrinsically superior *CAD1* gene in strain 6657-9B (see also figure 23), or if a further determinant exists which influences the mitochondrially mediated effect.

I propose experiments, which should provide rewarding insights pertaining to the issues raised in this section. First, the introduction of the *kar1* allele into a *CAD1* strain. Yeast strains, which are *kar1*, are defective in nuclear fusion i.e. in karyogamy (Conde 1976). When *kar1* and *KAR1* strains are crossed, the resulting zygotes are simultaneously heterokaryons and hetero-

plasmons or cytoductants. They bud off haploids with a nucleus from either parent and a cytoplasm from both parents. The rapid establishment of a homoplasmic state should then allow to closely examine the mitochondrial modulation of specific activity in a number of genetically diverse mitochondrial backgrounds.

A second set of promising experiments would be a screening for the specific mtDNA sequences mediating in the observed effects. A straightforward approach would be to select for a cad^+ phenotype in *CAD1 petites*. The purification and amplification of DNA sequences associated with the process of formation of ρ^- mitochondria would greatly simplify a subsequent analysis.

4.3.3. Summary and conclusions

I conclude that the *CAD1* gene is a dominant gene. It is encoded in the nucleus. I propose a distinct, mitochondrially mediated modulating effect on specific activity as responsible for the manifestation of cad^- phenotype in *CAD1* strains observed in some cytoplasmatic genetic backgrounds.

4.4. Isolation and characterization of the *CAD1* gene

4.4.1. Summary of results

The *S. cerevisiae* *CAD1* gene was isolated from a yeast genomic library by complementation. Four *cad*⁺ transformants were obtained. A 5.1kb *Hind*III fragment which conferred *cad*⁺ phenotype on *cad*⁻ strains was identified after subcloning. This fragment restored resistance to *t*-cinnamic acid in all *cad1* strains tested. Enzyme activity in transformed strains was elevated seven- to seventeen-fold over wild type levels. A 1.6kn putative *CAD1* transcript was identified after Northern hybridization. A DNA probe containing the *CAD1* gene hybridizes to *S. cerevisiae* chromosome II.

4.4.2. Discussion

The isolation of the *CAD1* gene is a further example of gene cloning by complementation in yeast. Early examples for this approach are the cloning of the *CAN1* (Broach 1979), *CDC10* (Clarke 1980) and *CDC28* (Nasmyth 1980) genes. The relative ease of obtaining mutants in yeast as well as the fact that most yeast genes are not interrupted by introns, render this approach the method of choice to isolate genes in yeast. Of the several hundred yeast genes cloned to date, only seventeen contain introns (Fink 1987), thus making cDNA cloning unnecessary for recombinant DNA research in yeast.

The transformation procedure used in the isolation of the *CAD1* gene gave rise to spurious results on two occasions. Further to the recombinant molecules carrying the *LEU2* and *CAD1* genes, other plasmid species were co-isolated during plasmid rescue into *E. coli*. In the latter yeast transformation, these plasmids were not the result of recombinatory rearrangements, because they were not related to the plasmids carrying *CAD1*. The results observed can be explained best as double transformations. Hicks (1979) has shown that a particular fraction of the protoplast population becomes preferentially competent after Ca^{2+} treatment. The simultaneous transformation of one protoplast with two different plasmids occurs at a frequency much higher than would be expected from the frequencies of independent transformation by the plasmids separately.

The probability of double transformation was further increased by the high DNA concentration used for transformation. In the transformation procedure used in this work, 1 μ g plasmid DNA was added per 5×10^7 protoplasts, whereas Beggs (1978) transformed with 1 μ g plasmid DNA per

1×10^9 protoplasts, Hinnen (1978) added $1 \mu\text{g}$ plasmid DNA per 3×10^8 protoplasts and Gerbaud (1979) transformed with $1 \mu\text{g}$ plasmid DNA per 6×10^7 protoplasts. With the transformation conditions used in this work, a large fraction of all transformants were probably multiply transformed protoplasts.

The *CAD1* gene could not be isolated by direct selection, that is, by plating the transformation mix on malt extract medium with sorbitol and *t*-cinnamic acid added. The clones that grew under these conditions amounted to approximately 10% of the number of transformants obtained under identical transformation conditions when selection was based on the complementation of an auxotrophic marker. However, the directly selected cad^+ clones did not harbor a recombinant plasmid and were identified as false positives. This necessitated a two-step selection procedure to isolate *CAD1*. The best explanation for the origin of these false positives is that they are polyploids that arose by protoplast fusion in the course of the transformation procedure.

The yeast transformation protocol is based on a method originally developed for protoplast fusion (Van Solingen 1977). Protoplast fusion also occurs in the course of yeast transformation. Hicks *et.al.* (1979) have shown that up to 25% of the transformed clones were polyploid with equal proportions of diploids and triploids; Karpova *et.al.* (1984) find that up to 75% of their transformants are polyploid. Ploidy was analyzed genetically, so that degrees of ploidy greater than triploids could not be analyzed which, however, does not preclude their existence. In the light of the pronounced threshold effects towards *t*-cinnamic acid sensitivity discussed in section 4.3.2., I expect that the existence of additional copies of *cad1* in polyploids should suffice to make these phenotypically cad^+ provided that selection is not very severe. Based on these observations, a procedure for the selection of polyploids generated by protoplast fusion could be developed.

The seven- to seventeen-fold increase in *t*-cinnamic acid decarboxylase activity observed in transformed cells compared to wild type cells is of a magnitude similar to the increases reported elsewhere. Gerbaud *et.al.* (1979) observe an elevation of orotidine 5'-monophosphate decarboxylase activity between 10- and 38-fold over wild type levels in transformed cells. The variance in the increase was vector and strain specific. Kuranda and Robbins (1987) find a 22-fold elevation over wild type levels of α -mannosidase activity in transformed cells with YEp24 used as vector; the increase is 10-fold in the case of exoglucanase activity. Chen and Hitzeman (1987)

find an 8.5-fold increase in 3-phosphoglycerate kinase levels in transformed cells, with an estimated copy number of the 2 μ based vector of 20 to 30. Arrese *et.al.* (1983) observe a 24- to 28-fold elevation of δ -aminolevulinic acid synthase activity when transformed cells are compared with wild type. This overview shows that the increase in enzyme activity observed in this work compares well with the data obtained in other investigations.

The high level of background observed in the hybridization experiments requires the repetition of these experiments in order to corroborate the conclusions regarding the size of the transcript and the chromosomal location of the *CAD1* gene. There is no satisfactory explanation for the spurious signal observed in the Southern blot when *LEU2* was included as positive control. The migration distance observed for the putative *CAD1* transcript may not reflect its authentic size with great accuracy because of interference caused by the high charge density in the immediate vicinity in the gel due to the large amounts of rRNA present. The use of poly(A)⁺ RNA instead of total RNA should yield more precise results for the determination of transcript size.

The transcript length of approximately 1600 nucleotides allows a closer delimitation of the exact location of the *CAD1* gene and its regulatory elements on the 5.1kb *Hind*III fragment. The observation that the *Bam*HI fragments present on pPD111, pPD112, pPD115 and pPD116 do not complement *cad1* permits the conclusion that the *Bam*HI restriction site present on the 5.1kb *Hind*III fragment is located within the regulatory elements or the coding sequence of *CAD1*. Even if the *Bam*HI site were physically located at the extreme ends of the *CAD1* sequence, one can assume, that the gene is not located on the 1.2kb *Bcl*I-*Hind*III subfragment of the 5.1kb *Hind*III fragment.

An estimate of the upper limit of the mass of the *t*-cinnamic acid decarboxylase molecule is possible now that the size of the putative *CAD1* transcript is known. Most yeast mRNAs have a 3'poly(A) tail (McLaughlin 1973) and a 5'cap (Sripati 1976). In *S. cerevisiae*, the 3'poly(A) tail is generally 50 nucleotides long (Santiago 1987). The 5'untranslated sequence is generally 40 to 80 nucleotides long with the extremes being between 3 and 742 nucleotides (Kozak 1983). A conservative estimate of the size of the coding sequence would be 1500 nucleotides. This would code for a protein with a mass of 55 kilodalton, assuming 110 daltons as the average mass of an amino acid. The yeast gene for elongation factor 3 (EF3) provides an example for the tenability of the assumption, that the total num-

ber of 5' and 3' untranslated nucleotides in yeast mRNA can be around 100. The 125 kilodalton EF3 protein requires a coding capacity of 3.4kb; the EF3 mRNA is 3.5kb in size (Qin 1987).

4.4.3. Summary and conclusions

The *S. cerevisiae* *CAD1* gene was cloned on a 5.1kb *Hind*III fragment. The regulatory elements and the coding sequences for the gene are probably located on a 3.9kb *Bcl*I-*Hind*III subfragment. The upper limit for the mass of the *t*-cinnamic acid decarboxylase polypeptide is 55 kilodalton. The distinct effect of gene dosage on the cad^+ phenotype suggest that this gene could be used as a marker for ploidy in protoplast fusion experiments.

4.5. Expression of the *CAD1* gene

4.5.1. Summary of results

The yeast *CAD1* gene is expressed in batch aerobic growth in complete or minimal medium. The best carbon source under these conditions is glucose, followed by ethanol and glycerol. In a batch fermentation in complete medium, the time course of expression follows exponential kinetics during growth of the cells on glucose. After complete consumption of glucose a doubling of specific activity is observed. This high-level expression decays only slowly. There is no indication of catabolite repression of *CAD1* or of catabolite inactivation of the enzyme. The *CAD1* gene is not expressed in *petites*, this is regulated at the level of transcription. The *S. cerevisiae CAD1* gene is expressed in *S. pombe*, albeit very inefficiently. In *E. coli*, growth on media with *t*-cinnamic acid is negatively influenced by the presence of plasmids encoding the yeast *CAD1* gene.

4.5.2. Discussion

CAD1 expression in *S. cerevisiae*

The data presented in table 6 does not permit the definite conclusion, that glucose is the best carbon source for expression of the *CAD1* gene. These results were obtained in batch growth experiments performed in Erlenmeyer flasks with baffles, with no attempt made to determine the growth stage of the culture. The results summarized in figure 32 show, however, that the increase in specific activity of the enzyme is not steady in the course of growth. It is therefore not possible to draw an unequivocal conclusion about the relative activities of *t*-cinnamic acid decarboxylase as a function of growth medium and carbon source without knowledge about the specific growth stage of the cultures.

It is beyond reasonable doubt that the enzyme is expressed constitutively. The complete medium employed in the fermentation is made up of acid-hydrolyzed yeast. The minimal medium utilized in the experiment described in figure 33 contains only mineral salts, carbon source and L-histidine. If the enzyme-catalyzed removal of a class of growth inhibitory compounds is the true function of *t*-cinnamic acid decarboxylase, then a potential inducer should be structurally related to the phenylacrylic acids. The presence of such a compound can reasonably be excluded in both experiments. However, the possibility of an higher level of inducible enzyme ac-

tivity supplementing a basal level of constitutive expression remains to be established.

The results obtained from the experiment summarized in figure 33 further allowed the following conclusions. Neither did catabolite repression influence the expression of *CAD1*, nor was there any indication of catabolite inactivation of *t*-cinnamic acid decarboxylase. After glucose was added to 2%, the specific activity remained essentially constant over a period of 180 minutes. During this time, the dry mass of the culture increased 1.5-fold. Koch and Friesen (1979), Chia and McLaughlin (1979) and Fuchs and Lacroute (1983) have observed mRNA half-lives of one to seventy minutes in *S. cerevisiae*. Santiago *et.al.* (1986) have determined the half-lives of 24 randomly selected mRNA species in yeast. They found two subpopulations in respect to lifetime, however, for both populations an inverse relationship between mRNA length and half-life existed. Assuming that the *CAD1* transcript belonged to the longer-lived population, an approximately 1.6kn long transcript would have a half-life of 60 minutes. If catabolite repression would influence the expression of the gene, then only 13% of *CAD1* mRNA should remain three hours after the addition of glucose. Assuming negligible enzyme degradation, the specific activity remaining after this period of time should drop to 66% of the level observed before due to dilution by growth. If catabolite inactivation were involved, the decrease in enzyme specific activity would be even more rapid. Gancedo (1971) e.g., showed fructose diphosphatase activity to be completely lost an hour after glucose addition to acetate-grown cells. However, no decrease of this magnitude in *t*-cinnamic acid decarboxylase specific activity was observed.

The difference in the kinetics of expression of *CAD1* observed in the experiments summarized in figures 32 and 33 cannot be explained yet. During growth on complete medium, the specific activity of the enzyme displayed an exponential increase, which roughly paralleled the growth curve; whereas growth on minimal medium resulted in no such increase. However, when compared to growth in complete medium, the culture in minimal medium was in a early growth stage and the amount of dry mass was still low. The rapid two-fold increase in specific activity observed during growth on yeast extract medium after the complete consumption of glucose was not observed during growth on minimal medium. It cannot be concluded yet whether this effect is a result of the coinciding pH minimum observed during fermentation or whether it is caused by some other parameter.

It is well conceivable, that this change in activity is the result of mitochondrial modulation of *CAD1* expression. When glucose is exhausted, the yeast cell can only derive energy equivalents from respiration. During the transition from fermentation to respiration, the structure (Stevens 1981) and respiratory ability (Fraenkel 1982) of the chondriome changes which may precipitate the observed rapid two-fold increase in specific activity.

The absence of enzyme activity and of *CAD1* specific mRNA in *petites* clearly demonstrated mitochondrial influence on expression of the *CAD1* gene. To my knowledge, this is the first report of a mitochondrial influence on gene expression of an enzyme most probably not involved in mitochondrial functions. Tzagoloff and Myers (1986) state that it is a safe assumption that mtDNA in general exerts little if any influence in regulating either transcription of nuclear *PET* genes or translation of their cytoplasmic mRNAs. *PET* genes would be the best candidates for this type of regulation, because they are by definition nuclear genes whose expression is required for the morphogenesis of respiratory competent mitochondria. They are termed *PET* because the allelic *pet* mutations confer a respiratory deficient phenotype comparable to a ρ^- mutation upon the affected cell.

Although mitochondrial DNA, that is a gene product of mtDNA, may exert no influence on the expression of nuclear genes the functional status of the *S. cerevisiae* mitochondrion definitely influences gene expression. The best example for this type of feed-back regulation is the influence of heme on the expression of a number of yeast genes. Heme forms the prosthetic group of cytochrome c and of other cytochromes. The genes encoding the proteins constituting the heme biosynthetic pathway are all located inside the nucleus. The heme biosynthetic pathway, however, is topologically interweaved between the cytosol and the membranes of the mitochondrion (Labbe-Bois 1983). In *S. cerevisiae*, heme is synthesized in sufficient amounts only when mitochondria are functional in the sense that they demonstrate respiratory proficiency (Mattoon 1979). Heme deficient cells lack the characteristic adsorption spectra for cytochromes a, a₃, b, c, and c₁ (Ephrussi 1950 and Gollub 1977). Furthermore, the apoproteins corresponding to several subunits of cytochrome oxidase (Saltzgaber-Müller 1978), catalase A and T (Woloszczuk 1980) and iso-1-cytochrome c (Matter 1982) are either absent or greatly reduced in heme deficient cells (Guarente 1983). In the presence of heme, the yeast *HAP1* gene product binds to one of the upstream activator sites (UAS₁) of the *CYC1* (iso-1-cytochrome c) gene (Pfeifer 1987a). This stimulates transcription of *CYC1* 200-fold (Guarente 1983).

I have discussed the heme driven transcriptional regulation of enzyme levels as an example how the functional status of the chondriome can influence and modulate gene expression. In this type of feed-back regulation, heme acts as a reporter molecule signalling the respiratory proficiency of the mitochondrion. Even if the function of the *CAD1* gene product were completely unrelated to any mitochondrial structure or function, which has yet to be conclusively determined, it is conceivable that the observed mitochondrial influence on the enzyme is mediated via heme or a comparable reporter molecule.

A possible link to mitochondrial structure or function may be the enzyme's location in the yeast cell. Considering the lipophilic nature of large parts of the substrate molecule, a membrane-associated location is quite probable. A membrane-associated location would greatly promote the decarboxylation rate by accelerating substrate diffusion in the yeast cell (Adam 1968). Possible locations that can easily be tested would be the plasma membrane or the mitochondrial membrane system.

I propose some experiments in order to resolve the issues raised. It should be established, whether *CAD1* is expressed under anaerobic conditions. A straightforward experiment to determine the cellular location of the *CAD1* gene product would be a rate-zonal density gradient centrifugation of gently lysed spheroplasts. After collecting the separated sub-cellular fractions, enzyme assays of *t*-cinnamic acid decarboxylase along with assays of other enzymes used as biochemical markers for yeast organelles (Schekman 1982) should reveal the cellular location of the *CAD1* gene product.

Further experiments are necessary to differentiate between two possible modes of mitochondrial modulation of the enzyme's activity. If a gene product of mtDNA is responsible for this effect, it should be detectable by the genetic tests proposed in section 4.3. If, however, modulation is not mediated by a polypeptide encoded by mtDNA but rather is a reflection of mitochondrial functional status, eg respiratory proficiency, then the influence on enzyme activity of experimental inhibition of various mitochondrial functions by antibiotics should be studied. The activity of *t*-cinnamic acid decarboxylase should be determined as a function of time under different inhibitory conditions alongside with the activity of other mitochondrial enzymes. Finally, the question, whether the regulatory elements of the *CAD1* gene possess any DNA sequence capable of interacting with a putative *trans*-acting regulatory polypeptide can rapidly be answered in a gel retardation assay (Fried 1981 and Garner 1981).

CAD1 gene expression in *Schizosaccharomyces pombe*

The underlying cause of the observed variance in *t*-cinnamic acid decarboxylase activity in different clones of transformed *S. pombe* is probably of complex nature. In the following section two probable causes, plasmid instability and aberrant gene expression, will be discussed.

Although *S. pombe* can easily be transformed in terms of efficiency by some 2 μ -based *S. cerevisiae* - *E. coli* shuttle vectors (Beach 1982a), this finding in itself was fortunate. The 2 μ *ori* sequence that is responsible for autonomous replication of 2 μ -based plasmids in *S. cerevisiae* does not function as replication origin in fission yeast (Gaillardin 1983). Instead, one of the inverted repeat sequences (IRS) present on the 2 μ plasmid suffices for autonomous replication in *S. pombe* (Gaillardin 1983).

However, even when this IRS is present, the plasmids are very unstable after being introduced into *S. pombe*. Apparently, the partitioning of plasmids into daughter cells in the course of mitotic division is very inefficient. Gaillardin *et.al.* (1983) report losses of 5 to 7% per generation on non-selective medium; Beach *et.al.* (1982a) find that after one cycle of subculturing (10-15 generations) under selective conditions, pBD248', which contains the same eukaryotic sequences as pDB262 (Beach 1982b), is lost from 20% of the cells. Wright *et.al.* (1986) report that typically 30 to 60% of cells originally transformed with pDB262 retain the plasmid after propagation to saturation in a selective medium. A determination of copy number of 2 μ -based plasmids in *S. pombe* is futile under these circumstances.

The only indication of the average number of 2 μ derived plasmids present in transformed *S. pombe* are the few reports on mRNA levels deriving from transcription of plasmid-borne genes. Toda *et.al.* (1984) find a five-fold elevation in the mRNA levels of *S.pombe* α 2 tubulin in cells transformed with a pDB248' based plasmid when compared to wild type levels; however, no difference was found in α 1 tubulin mRNA levels when this *S. pombe* gene was present on a plasmid. Russel (1983) reported a three to five-fold increase in fission yeast alcohol dehydrogenase mRNA levels in *S. pombe* *leu1-32* cells transformed with a 2 μ -based vector carrying *S. pombe* *ADH* when compared to the untransformed wild type.

A number of *S. cerevisiae* genes have been expressed in *S. pombe*. The *S. cerevisiae* α -mannosidase, exoglucanase and endochitinase genes have been expressed in *S. pombe* (Kuranda 1987). The *S. cerevisiae* *URA3* gene complements the *S. pombe* *ura4* gene (Gaillardin 1983). The *S. pombe*

cdc2 gene can be complemented by the *S. cerevisiae* *CDC28* gene (Beach 1982b); *S. cerevisiae* *URA1* and *PPR1* are expressed in fission yeast (Losson 1985) and, finally, the *S. cerevisiae* *ADC1* and *ADR2* genes are expressed in *S. pombe* (Russel 1983).

However, *S. cerevisiae* genes are expressed inefficiently in *S. pombe* due to differences in their mechanisms of gene expression. Most is known about differences in the initiation step of transcription. Russel (1985) has shown, that the *S. pombe* triose phosphate isomerase initiates from a start point different from that in *S. cerevisiae*. Losson *et.al.* (1985) have shown, that the 5' ends of the mRNA molecules transcribed in *S. pombe* from the *S. cerevisiae* *URA1* and *URA3* genes map upstream from the initiation sites used in *S. cerevisiae*. Russel (1983) has shown, that the *S. cerevisiae* *ADC1* and *ADR2* genes, when introduced into fission yeast, have transcriptional start points nearer to the Goldberg Hogness sequence (TATAAATA) than it is the case when being transcribed in *S. cerevisiae*. The inverse also applies; when *S. pombe* genes are transcribed in *S. cerevisiae*, the transcriptional start points are nearer to the start codon than it is the case when they are transcribed in fission yeast (Russel 1983). In *S. pombe*, the start site of transcription lies 35-45 nucleotides downstream of the TATA box (Russel 1986). This is comparable to the situation observed for vertebrate genes, where the distance between the TATA box and the transcriptional start point is always 25-30 nucleotides (Breathnach 1981). In *S. cerevisiae*, however, a much greater variability exists, distances from 35 to 180 nucleotides have been observed (Sentenac 1982)

Summarizing, both plasmid instability and inefficient gene expression are probably causative for the low and concomitantly highly variable level of expression of *S. cerevisiae* *CAD1* in the heterologous host *S. pombe*. It is very probable, that the activity measured in the CAD assay derives from a longer transcript than in *S. cerevisiae*.

CAD1 expression in *Escherichia coli*

The results summarized in table 8 show, that the presence of the yeast *CAD1* gene on a plasmid in transformed *E. coli* has deleterious effects on this procaryotic host. Although enzyme assays of transformed *E. coli* were not performed, it is quite probable that the gene is expressed in this host. However, it cannot be completely excluded that the observed effect is due to a further transcript originating from this fragment of yeast chromosomal DNA.

Many of the yeast genes cloned early on were isolated by their ability to complement *E. coli* mutants, for which gene expression is a prerequisite. The yeast *HIS3*, *LEU2*, *URA3*, *TRP1*, *TRP5* and *ARG4* genes were first cloned this way (Beggs 1981). It would be rewarding to perform the experiment summarized in table 8 again with an insert whittled down to the *CAD1* gene itself.

4.5.3. Summary and Conclusions

The *S. cerevisiae* *CAD1* gene is expressed constitutively in minimal and complex media. Glucose repression was not observed. Different levels of expression in the course of batch growth are probably influenced by the functional status of the mitochondrion. Mitochondrial influence is clearly evident in the demonstrated absence of any *CAD1* transcript in *petites*. The low and highly variable activity of *t*-cinnamic acid decarboxylase observed in *S. pombe* are probably due to the combined effects of plasmid instability and inefficient expression of the heterologous *CAD1* gene. *CAD1* is probably expressed in *E. coli*.

4.6. Analysis of the *CAD1* to *lacZ* fusions

4.6.1. Summary of results

Fusions of yeast chromosomal DNA to an *E. coli* *lacZ* gene were generated *in-vivo*. 14 plasmids carrying sequences from both strands of the *S. cerevisiae* 5.1kb *HindIII* fragment capable of directing the transcription of the downstream *lacZ* gene were analyzed in detail. Four plasmids were identified which may carry an authentic *CAD1*'*lacZ* gene fusion. No viable *petites* could be obtained from clones harboring deletion plasmids still capable of high-level *CAD1* expression.

4.6.2. Discussion

Gene fusions were made to facilitate analysis of the expression and regulation of the *CAD1* gene. The generation of a fused gene resulting in a bifunctional polypeptide was perceived to facilitate the determination of the intracellular location to which the *CAD1* gene product is targeted.

Gene fusions to *lacZ* have widely been used to analyze the expression of β -galactosidase as directed by yeast regulatory elements. After gene fusion to *lacZ*, Cunin *et.al.* (1986) were able to analyze the very complex regulation of expression of the *CAR1* gene. The analysis of the translational regulation of expression of the regulatory gene *GCN4* was made possible by gene fusions to *lacZ* (Thireos 1984 and Hinnebusch 1984). The demonstration that heme regulates the transcription of *CYC1* via an upstream activation site (Guarente 1983) as well as the demonstration, that these UAS are active when inverted, but not when placed downstream of *CYC1* (Guarente 1984) und thus do not qualify as enhancers, was possible after fusion of *CYC1* to *lacZ*. With the help of *lacZ* fusions, the targeting of the *MATa2* gene product to the nucleus (Hall 1984) and of a 70kd protein to the yeast mitochondrial outer membrane (Hase 1986) could be demonstrated. These examples show that gene fusions to *lacZ* can be utilized to address a wide range of problems, in fact some problems only become tractable with this approach (see, for example Bowen 1984).

The *in-vivo* approach for deletion formation was chosen in this work because no details on the location of the *CAD1* gene on the 5.1kb *HindIII* fragment and on the direction of it's transcription were known at that time. The *in-vivo* generation in *E. coli* of deletions for fusing yeast DNA sequences to a truncated *lacZ* gene was first described by Rose *et.al.* (1981). These authors stated that most deletion events are catalyzed by the

recombination system of *E. coli*, so that the host should be recombination proficient (Rose 1983). This approach was also followed in this work.

However, it has been demonstrated, that deletion formation is *recA* independent (Franklin 1967 and DasGupta 1987). Shen and Huang (1986) have determined a minimum length of 23-27 homologous base pairs required for wild type homologous recombination, that is recognition of homology ascribed to *recA* and recombination proceeding via the *recBCD* pathway. This quite rigorous requirement excludes participation of *recA* in deletion formation, as the probability of a sequence homology on the 5.1kb fragment of yeast DNA to any random 23bp stretch in the 105 base pairs preceding the truncated *lacZ* gene on YE ϕ Z100 is approximately 1.5×10^{-12} .

DasGupta *et.al.* (1987) have shown that direct and inverted repeats with as few as four base pairs promote deletion events with mechanistic differences very probable in the formation of large and small deletions. These authors hypothesize that most deletions arise by replication errors and show a profound effect of insertion site on the frequency of deletion. The investigations on the mechanism of deletion formation show that the distribution of *in-vivo* generated deletion end-points will at best be semi-random. Most of the deletions formed will not be recovered by the system used in this work due to the inability of the yeast sequence joined to *lacZ* to direct the latter gene's expression.

I expect the *in-vivo* generated deletion junctions to be clustered around either the 5'noncoding regulatory elements or the 3'transcription termination region due to inherent structural features making these more susceptible to deletion. The 5'noncoding sequences of *TRP1*, *HIS3*, *HIS4* and *TRP5* have been shown to possess a region of dyad symmetry (Dobson 1983); the UAS of *CYC1* and *CYC7* possess a number of direct repeats (Pfeifer 1987a and 1987b). Henikoff *et.al.* (1983) have suggested the involvement of a repeated eight base pair sequence in termination of transcription and Umek and Kowalski (1987) have shown that a termination sequence from yeast adopts a non-B DNA conformation. The structural motifs described are preferentially deletion-prone because they interfere with DNA replication (Kornberg 1980 and 1982; and DasGupta 1987). The 105bp *HindIII*-*EcoRI* fragment present immediately upstream of the truncated *lacZ* gene contains many sequences partially or completely homologous to the sequences described above (Hartley 1980), and thus could provide complementary sequences promoting the formation of hairpin structures.

The results in this work confirm the observations and predictions described above. None of the deletion plasmids analyzed in detail had retained any of the restriction sites present between the 5.1kb *Hind*III fragment and the coding sequence of the truncated *lacZ* gene, leaving the junction somewhat ill-defined. The high density of sequences present on the 5.1kb *Hind*III fragment that are able to direct transcription of the *lacZ* gene in *E. coli* is quite surprising. If the observation of β -galactosidase activity in *S. cerevisiae* is a sufficient and valid criterion for a true fusion between a yeast gene and *lacZ* (Rose 1983), then there are three yeast genes present on the 5.1kb *Hind*III fragment. Candidates for fusions of regulatory elements of yeast not normally transcribed in *S. cerevisiae* but capable of directing *lacZ* transcription in *E. coli* are plasmids pZYCr54, pZYCs14 and pZYDs35 for fusions to 5'elements and plasmids pZYDs47, pZYDs49 and pZYDs33 for fusions to 3'elements. The activity of β -galactosidase tended to be higher in *S. cerevisiae* when the junctions were nearer to the amino terminal part of the putative gene (compare eg. pZYCs20 to pZYCs12). This confirms observations made by Rose *et.al.* (1983).

The distance spanned between pZYDs35 and pZYDs44 is approximately 1.5kb which compares well with the size of the transcript observed in the Northern blot. It remains to be established unequivocally by analysis of proteins whether pZYDs51, pZYDs25, pZYDs37 and pZYDs44 carry fusions that code for bi-functional polypeptides. From the data obtained in this work, I conclude that the sequences regulating *CAD1* gene expression will be physically located in the immediate vicinity of the *Stu*I and the neighboring *Sca*I restriction enzyme recognition sites. The coding sequence of *CAD1* is predicted to terminate upstream of the *Xmn*I site.

The inability to obtain viable *petites* harboring plasmids expressing high levels of *t*-cinnamic acid decarboxylase cannot be explained. This observation does not parallel the earlier one, that the yeast transformants originally picked could not be propagated on media with 2% glucose, because the cultures were plated on a minimal medium containing 2% glycerol and 0.2% glucose.

4.6.3. Summary and conclusions

Fusions of yeast chromosomal DNA to an *E. coli* *lacZ* gene were generated *in-vivo*. In contrast to the assumptions of Rose (1983), deletion formation is probably not *recA* catalyzed (DasGupta 1987). The use of a *recA*⁻ strain for generation of deletion plasmids would have greatly facilitated

subsequent analysis, because the observed frequency of rearrangements would have been far smaller. Of the 14 plasmids carrying sequences from both strands of the *S. cerevisiae* 5.1kb *Hind*III fragment capable of directing the transcription of the downstream *lacZ* gene, eight constitute genuine gene fusions. Four plasmids were identified which may carry an authentic *CAD1*'*lacZ* gene fusion. The fusion junctions of the remaining plasmids are probably localized in regulatory elements not normally transcribed in *S. cerevisiae*. The extent of the *CAD1* gene could be delimited more precisely. It is most probably located between the *Xmn*I and the next *Sca*I site. The inability to obtain viable *petites* harboring deletion plasmids still capable of high-level *CAD1* expression could not be explained.

5. Summary and conclusions

1. Growth of wild type yeast cells was inhibited by *t*-cinnamic acid. The degree of inhibition was pH dependent and inhibition increased as pH was lowered. The presence of glucose in the medium alleviated growth inhibition. *t*-Cinnamic acid interfered with mass flux across the membrane. Growth inhibition was probably caused by interference with cellular energy metabolism as well as with transport across the cytoplasmic membrane. The models proposed by Krebs *et.al.* (1983) and Freese *et.al.* (1973) were insufficient to explain the observed effects.

2. *S. cerevisiae* decarboxylated *t*-cinnamic acid stereospecifically to styrene. Phenylacrylic acids that occur naturally as intermediates in the phenylpropanoid pathways of higher plants were decarboxylated to the corresponding substituted styrenes. The proposed function of this enzyme in the natural habitat of yeast is the detoxification of growth inhibitory compounds produced by higher plants. Analogs substituted with a nitro group at the 3- and 4- positions of the aryl moiety functioned as substrates. The enzymatic reaction was inhibited by the presence of complexogenic carboxylate anions. 3-phenylpropionic acid and 2-phenylethanol were identified as potent inhibitors. Inhibition by 3-phenylpropionic acid corroborated previous evidence for a two step addition-elimination reaction sequence. *Fusarium oxysporum* also possessed CAD activity, whereas *Schizosaccharomyces pombe* did not.

3. Stable, nonconditional and respiratory proficient cad^- mutants were obtained after nitrosoguanidine mutagenesis. The different mutant alleles analyzed all belonged to one complementation group. The wild type allele was dominant over the mutant alleles tested. The genetic information for *t*-cinnamic acid decarboxylase was found to be encoded in the nucleus. The manifestation of cad^- phenotype in *CAD1* strains observed in some cytoplasmic genetic backgrounds was explained best as mitochondrially mediated modulation of observable enzyme activity.

4. The *S. cerevisiae* *CAD1* gene was isolated from a yeast genomic library by complementation. Four cad^+ transformants were obtained. A 5.1kb *Hind*III fragment, which conferred cad^+ phenotype on all *cad1* strains tested, was identified after subcloning. Enzyme activity in transformed strains was elevated seven- to seventeen-fold over wild type levels. A 1.6kb putative *CAD1* transcript was identified after Northern hybridization. The

regulatory elements and the coding sequences for the gene were assumed to be located on a 3.9kb *BclI-HindIII* subfragment. The upper limit for the mass of the *t*-cinnamic acid decarboxylase polypeptide was estimated to be 55 kilodalton. The distinct effect of gene dosage on the *cad*⁺ phenotype suggested that this gene could be used as a marker for ploidy in protoplast fusion experiments. A DNA probe containing the *CAD1* gene hybridized to *S. cerevisiae* chromosome II.

5. The *S. cerevisiae CAD1* gene was expressed constitutively in batch aerobic growth in complete or minimal medium. The best carbon source under these conditions was glucose, followed by ethanol and glycerol. The functional status of the mitochondrion was assumed to influence expression levels in the course of batch growth. There was no indication of catabolite repression of *CAD1* or of catabolite inactivation of the enzyme. The *CAD1* gene was not expressed in *petites*, this was regulated at the level of transcription. The low and highly variable activity of *t*-cinnamic acid decarboxylase observed in *S. pombe* was probably due to the combined effects of plasmid instability and inefficient expression of the heterologous *CAD1* gene. Growth of *E. coli* on media with *t*-cinnamic acid was negatively influenced by the presence of plasmids encoding the yeast *CAD1* gene.

6. Fusions of yeast DNA to an *E. coli lacZ* gene were generated *in-vivo*. 14 plasmids carrying sequences from both strands of the *S. cerevisiae* 5.1kb *HindIII* fragment were identified which were capable of directing the transcription of the downstream *lacZ* gene in *E. coli*. Eight of these plasmids contained genuine gene fusions as judged by their ability to direct the expression of β -galactosidase in yeast. Four plasmids were identified which may carry a true *CAD1-lacZ* gene fusion. The fusion junctions of the remaining plasmids were probably localized in regulatory elements not normally transcribed in *S. cerevisiae*. The *CAD1* gene was assumed to be located between the *XmnI* site and the next *ScaI* site. The coding strand for *CAD1* was identified as the one, on which the recognition sites for the following restriction enzymes appear in the order 5' *PvuII* *BamHI* *KpnI* 3'. No viable *petites* could be obtained from clones harboring deletion plasmids still capable of high-level *CAD1* expression.

Abbreviations

ARS	: autonomously replicating sequence
BCIP	: 5-bromo-4-chloro-3-indolylphosphate
CAAD	: enzyme assay of <i>t</i> -cinnamic acid decarboxylase, with a specified phenylacrylic acid as substrate
CAD	: enzyme assay of <i>t</i> -cinnamic acid decarboxylase, with <i>t</i> -cinnamate as substrate
cad ⁺	: <i>t</i> -cinnamic acid resistant phenotype
cad ⁻	: <i>t</i> -cinnamic acid sensitive phenotype
<i>cadI</i>	: yeast with mutated allele of the <i>t</i> -cinnamic acid decarboxylase gene
<i>CAD1</i>	: yeast with wild type allele of the <i>t</i> -cinnamic acid decarboxylase gene
cDNA	: complementary DNA
CDTA	: <i>trans</i> -1,2-diaminocyclohexane- <i>N,N,N',N'</i> ,-tetraacetic acid
dm	: dry mass
EGTA	: ethyleneglycol-bis(β -aminoethyl ether)- <i>N,N,N',N'</i> ,-tetraacetic acid
epr	: electron paramagnetic resonance
IRS	: inverted repeat sequence
kb	: thousand base pairs
kd	: kilodalton
kn	: thousand nucleotides
krpm	: thousand rotations per minute
LN ₂	: liquid nitrogen
m.o.i.	: multiplicity of infection
MNNG	: 1-methyl-3-nitro-1-nitrosoguanidine
mRNA	: messenger RNA
mtDNA	: mitochondrial DNA
NBT	: nitro blue tetrazolium
NMR	: nuclear magnetic resonance
ONPG	: <i>o</i> -nitrophenyl- β -D-galactopyranoside
PMSF	: phenylmethylsulfonylfluoride
rpm	: rotations per minute
Tris	: 2-amino-2(hydroxymethyl)-1,3-propandiol
tRNA	: transfer RNA
UAS	: upstream activation sequence

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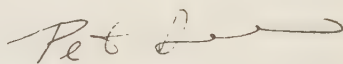
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Hiermit versichere ich, daß ich die Arbeit selbständig verfasst und nur die angegebenen Hilfsmittel benutzt habe.

A handwritten signature in dark ink, appearing to read 'Pet. D.' followed by a long horizontal flourish.

CURRICULUM VITAE

- 12.28.1953 Born in Manila, Philippines as only child of Ursula Doerner and Wolfgang Doerner. Nationality: German
- 1960-1966 Primary school: Jose Abad Santos School, Quezon City and American School, Makati, Philippines.
- 1966-1968 Institut Montana, Zug, Switzerland
- 1968-1973 Gymnasium Gutenbergschule, Wiesbaden, FRG
- 1973-1974 Military duty
- 1974-1979 Studies at the University of Oldenburg; courses taken: Microbiology, Applied Biology, Zoology, Botany, Biochemistry, Biotechnology, Chemistry, Physics, Mathematics.
- 1980 Research at the "Gesellschaft für Biotechnologische Forschung", Brunswick, FRG
- 1981 Graduation from the University of Oldenburg
- 1981 Eight weeks with CHEMAP AG Männedorf, Switzerland; during which I co-supervised the shakedown run of a bakers' yeast plant in Dar-el-Beida, Algeria.
- 1981 Affiliation with the University of Oldenburg, at the Chair of Applied Biology, as research/teaching assistant.
- 1986 Final experiments and completion of the PhD thesis.

PUBLICATIONS AND LECTURES

- 1) Doerner, P. *et.al.*: Direct Measurement of Volatile Compounds in the liquid-phase of an Acetone-Butanol Fermentation by Mass Spectrometry. Poster at the Second European Congress of Biotechnology, Eastbourne, GB 1981
- 2) Doerner, P. *et.al.*: Die Messung von flüchtigen Komponenten bei der Aceton-Butanol Fermentation mit Hilfe eines Quadrupol Massenspektrometers; Lecture, given at the "Interne Arbeitstagung des GVC Fachausschusses Bioverfahrenstechnik", 1982
- 3) Doerner, P. *et.al.*: Process Analysis of the Acetone-Butanol Fermentation by Quadrupole Mass Spectrometry. *Biotechnology Letters* 4 (1982) 557
- 4) Doerner, P. *et.al.*: Process Analysis of the Acetone-Butanol Fermentation by Quadrupole Mass Spectrometry. p 81 in: The Acetone-Butanol Fermentation & related topics, Eds: J.D. Bu'Lock and A.J. Bu'Lock ; Science and Technology Letters, Kew, Surrey 1983
- 5) Doerner, P. *et.al.*: The Cinnamic Acid Decarboxylase of *S. cerevisiae*. Poster at the 13th Int. Conf. on Yeast Genetics and Molecular Biology, 1986

